Increase in the predicted mRNA stability of certain SARS CoV-2 mutant spike proteins compared to wild type may pose potential risk to vaccines.

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Emergence of mutant variants in severe acute respiratory syndrome coronavirus-2 (SARS CoV-2) has been evident in the past two years (2019 to 2021). Irrespective of the origin of mutations, these mutant variants caused a great liability to human life with high morbidity rates across the world. Minute changes in the surface topology of the viral spike protein may cause significant changes in its epitope and may contribute to the failure of the current vaccines. In this study, we generated mutations in a systematic way throughout the receptor binding domain (RBD) of the spike protein and calculated the corresponding mRNA stability of the mutants. Our results indicate that more than 15 mutant variants have higher mRNA stability when compared to the wild type spike protein mRNA. By taking the predicted stability of mRNA as a guide we accessed the potential risk of epitope changes in such mutants that may cause risk to the existing vaccines. Our findings suggest that there is a potential risk for epitope changes that has to be evaluated in the future.

Keywords: SARS CoV-2, Vaccines, mutants, COVID-19, bioinformatics, mRNA stability.

Severe acute respiratory syndrome (SARS) caused by coronavirus-2 (CoV-2) has created a pandemic in recent years claiming a high number of human lives across the world (1). The first wave of infections in the years 2019-2020 (2) predominantly contained wild type strains of SARS CoV-2 but the second wave of infections in the year 2021 (3) was dominated by the delta variant. As of the date in December 2021, the omicron strain is the dominant variant of SARS CoV-2 (4) and is a threat for the third wave of infections. Amid the pandemic (5, 6), either with or without appropriate clinical trials (6-9), vaccines were massively produced and distributed around the world to protect the humans against the novel coronavirus disease - 2019 (COVID-19). How -ever, these vaccines were mainly focused on the wild type strains of the SARS CoV-2 and may offer a limited protection against the mutant variants of the virus due to mutations in the viral spike protein epitope that is recognized by the host antibodies generated through vaccination. Especially, the vaccine may not protect against the highly mutated omicron variant. Fortunately, the omicron variant with severe mutations in its spike protein may not have as much viral fitness as the wild type and other strains that may make it less dangerous. In order to encounter highly mutated strains (11), newly designed vaccines that trigger antibodies which can recognize the mutant variants of SARS CoV-2 are urgently needed. In this study, we considered 600 bases from the receptor binding domain (RBD) of spike protein mRNA and performed mutations systematically to understand their effect on overall stability and the secondary structure of the mutant mRNAs that may give us clues for vaccine design.

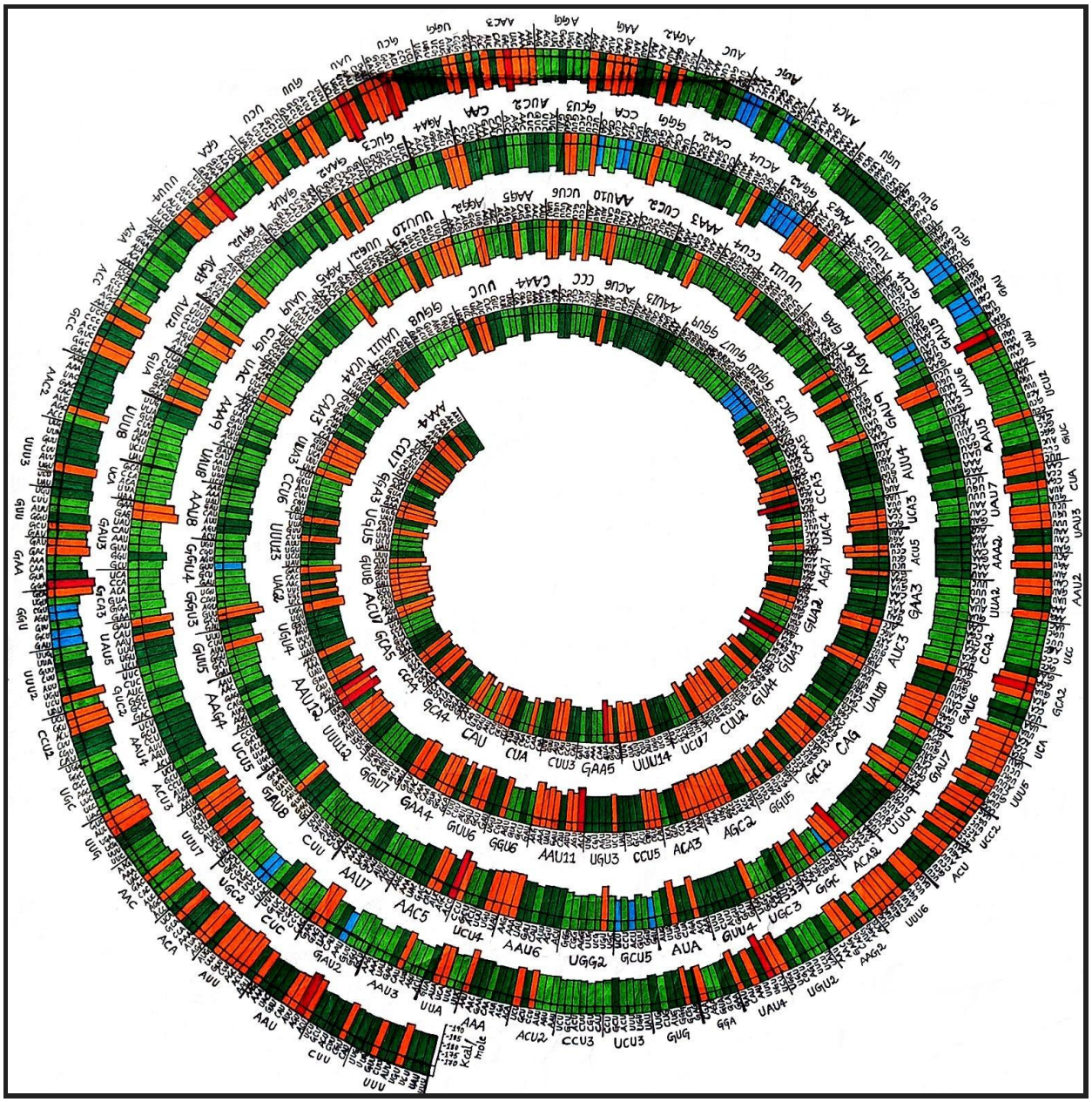

Figure 1. Hand-drawn RBD-mRNA free energy Spiral-plot.

Materials and Methods: RNAfold secondary structure prediction analysis was performed as described previously by Vissapragada *et al*. 2021 (12). Briefly, the wild type DNA sequence of SARS CoV-2 spike protein was taken from the National Center for Biotechnology Information with accession number: NC_045512.2.

The sequence was submitted to the RNAfold web server (http://rna.tbi.univie.ac.at/) as it is to obtain the predicted secondary structure along with the ensemble minimum free energy and the ensemble diversity (13-15). In the case of mutant variants, the entire sequence taken from wild type was organized as triplet codons to generate all the possible combinations of base substitutions and then the mutant

sequence was submitted to the RNAfold web server. The secondary structures for each mRNA was downloaded as an image file to compare the mutants with wild type. The thermodynamic minimum free energy values for wild type and each systematically generated mutant were tabulated (Table S1) and were then used to generate the hand-drawn spiral-plot (Figure 1).

Results and Discussion: In this study we evaluated the mRNA secondary structure prediction of SARS CoV-2 spike protein mRNA and its relative ensemble stability analysis using RNAfold web server (http://rna.tbi.univie.ac.at/). The minimum free energy of the predicted secondary structure ensemble for the wild type spike protein RBD mRNA (12) was taken as a control and was compared to the minimum free energy of the mutant variants. The mutant variants were generated systematically to evaluate the impact of both the base substitution itself and the position of the base substitution within the RBD mRNA. Our analysis revealed that out of 600 bases sequence that we considered in this study from the spike protein RBD mRNA, 18 base substitutions at 17 different positions resulted in mutant mRNAs with relatively higher ensemble stability compared to the wild type RBD mRNA. Previously, we have determined the thermodynamic ensemble free energy of the wild type mRNA (-183.78 kcal/mol. obtained from the RNAfold server) (12). In this study, the wild type was considered as the control value with which the viral replication and fitness (as evidently seen by the high morbidity rates caused by COVID-19 across the world since 2019) was taken as 100%. Thermodynamic ensemble free energy values of mutant variants that were systematically generated in this study ranged from -177.11 kcal/mol. to -189.28 kcal/mol. (obtained from RNAfold server). These results suggest that certain mutant variants may have relatively more stable mRNA secondary structure ensembles compared to the other with respect to the wild type mRNA.

Generally the more negative the free energy value, the more stability is expected for the secondary structure ensemble. For example, the mutant variant with minimum free energy of -177.11 kcal/mol. is less stable compared to the wild type mRNA (-183.78 kcal/mol.) and the mutant variant with minimum free energy of -189.28 kcal/mol. is more stable compared to the wild type mRNA (-183.78 kcal/mol.). In order to analyze the data clearly and conveniently, we plotted the thermodynamic free energy values of wild type and all the mutant variants that were generated in this study in a spiral-plot as shown in Figure 1. In this spiral-plot, the thermodynamic free energy values were taken on the negative y-axis such that all the bars in the spiral-histogram are pointing downwards facing the center of the spiral. Color coding was given to the bars depending on the values for convenient interpretation. Dark green bars are either wild type values or close to wild type values, light green bars are less negative than wild type bars, light blue bars are further less negative than light green bars. On the other hand, orange color bars have more negative value than wild type and the red colored bars have the most negative values obtained in this study. Taken together, we identified 18 red bars at 17 positions all which have more stable secondary structure ensembles of their mRNA compared to the wild type. If the wild type was taken as 100% fit in replication and infection, then one can speculate the 18 mutant variants with relatively more stable mRNA secondary structure ensembles could have more viral fitness than the wild type. Such mutant variants may pose risk to the existing vaccines because these mutations in the mRNA sequence may lead to amino acid substitutions in the RBD of the mutant spike protein with altered epitopes. Additionally, we have

recently shown that changes in the epitope topology may not necessarily occur at the site of mutation but can also lead to off-site epitope changes (16).

SARS CoV-2 has an RNA-based genome that not only replicates directly to form new virions but also contributes to the translation of viral proteins with the help of host cell machinery such as the ribosomes, etc. The viral replication is mainly controlled by the viral RNA-dependent RNA polymerase (RdRP) which contains the 3'-exonuclease activity for proofreading the daughter RNA strand during replication. Hence it is not feasible in general for this virus to evolve fast by incorporating mutations into its genome during replication (17). However, drugs such as molnupiravir may induce mutations in the viral genome randomly giving rise to mutant variants that may or may not be viable in terms of both replication competency and infectivity. Random incorporation of C or U into the viral genome in the presence of molnupiravir may have positive or negative effects on the host depending on the secondary complications such as diabetes, etc. Usually the first round of vaccine design is done against the wild type virus that has 100% viral fitness (evident from the pandemic since 2019). Thus, the antibodies generated by the vaccinated hosts may not recognize the altered epitopes of the mutant variants resulting in decreased potency of the vaccine (18). In such cases, new vaccines are needed to encounter both the wild type and the mutant variants of the virus. In order to understand the feasibility and survival of the mutant strains, we focused on the mRNA stability of systematically generated mutants in the RBD of wild type spike protein mRNA. This analysis gives us most, if not all, of the possible base substitutions that give rise to the mutant variants. With the knowledge of systematically generated mutant variants, one can redesign the vaccines to make them more effective against wild type and mutants.

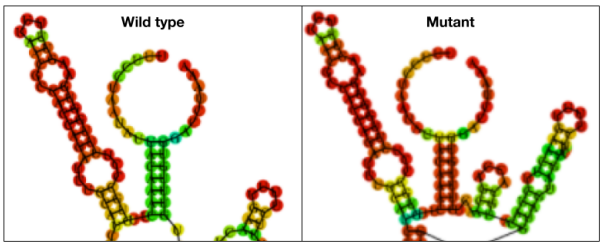

Figure 2. Changes in the RBD mRNA secondary structure.

Prediction of the secondary structure for wild type as well as mutant mRNAs of RBD helps us to analyze how the base substitutions are rearranging the base pairing within the secondary structure. RNAfold server was used to calculate the secondary structures of wild type and mutant RBD mRNAs. As shown in Figure 2, the mRNA secondary structures of the mutant variants indeed changed compared to the wild type. Typically gene expression and protein expression are controlled at transcription and translation levels, respectively. In the case of SARS CoV-2, due to the RNA genome of the virus, only translational control of viral protein expression is possible. It has been well known that the longer a particular mRNA molecule stays stable in the cytoplasm, the more protein molecules are translated by the ribosomes. One of the cellular strategies to regulate such uncontrolled protein translation is to degrade the mRNA by the exonucleases (19). However, if a particular mRNA molecule has a stable secondary structure, it may stay longer in the cytoplasm by escaping the exonuclease-mediated degradation giving rise to prolonged protein translation. It has been shown previously that virus induced mRNA degradation may occur some times to hack the host cell machinery effectively by the virus by rendering the host cell defense mechanisms dampened (20). To this end, we believe that if a particular mutant variant that was systematically generated in this study has an mRNA that shows relatively higher stability compared to the mRNA of the wild type then it is a safe assumption that this mutant will most likely have more viral fitness (>100%) than the wild type virus.

In this study, we identified 18 RBD mRNA secondary structure ensembles from the mutant variants compared to the wild type. Base substitutions in the systematically generated mutant variants of this study lead to epitope topology changes. This may or may not occur for all the base substitutions because a single amino acid may have multiple codons that may tolerate the base substitution and still insert the correct amino acid during protein translation. In such cases, the epitope would still be recognized by the antibodies generated by the current vaccines in the host. However, if the amino acid substitution takes place then epitope topology changes are most likely expected in the spike protein RBD. The effect of such change is two-fold, (i.) the virus may not be able to infect the new host cells at all due to loss of binding affinity to the host ACE-2 receptor due to altered topology of the spike protein RBD and (ii.) the virus may still infect new host cells either with decreased/ diminished fitness or with an enhanced fitness and infectivity.

The RNAfold server also provides the ensemble diversity number along with the ensemble thermodynamic free energy. In this study we did not consider the ensemble diversity because we did not make a direct correlation between the mRNA stability vs. viral fitness. This study is strictly focused on the variability in mRNA secondary structure in systematically generated mutant variants compared to the wild type. In future, we are going to extend this study to analyze the epitope topology of the mutant spike proteins in combination with the mRNA stability to establish a more robust correlation between the mutant mRNA stability and viral fitness. All future analyses will be published in the future issues of TCABSE-J.

Conclusion: Based on the current study, it is evident that the base substitutions in RBD mRNA sequence directly affects the thermodynamic ensemble free energy of the resulting mutant mRNA sequence compared to the wild type. Stable secondary structures of mRNA may lead to prolonged protein translation resulting in the abundance of viral proteins that can be packed as new virions which will infect the new host cells. Whether these mutations would enhance viral infectivity is a question that still has to be answered in future. Our ongoing research will shed light onto this in future.

Supplementary data: Supplementary data (Table S1) for this article is available online along with this article.

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