Epitope Identification and Designing a Potent Multi-epitope Vaccine Construct against SARS-CoV-2 Including the Emerging Variants

Sivasubramanian Srinivasan, Gracy Fathima Selvaraj, Vidya Gopalan, Padmapriya Padmanabhan, Kiruba Ramesh, Karthikeyan Govindan, Aswathi Chandran, Prabu Dhandapani1, Kaveri Krishnasamy, Satish Srinivas Kitambi2

Department of Virology, State Viral Research and Diagnostic Laboratory (VRDL), King Institute of Preventive Medicine and Research, 1Department of Microbiology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, Tamil Nadu, 2Department of Translational Sciences, Institute for Healthcare Education and Translational Sciences, Hyderabad, Telengana, India

Abstract

Introduction: The emergence of a novel coronavirus in China has turned into a SARS-CoV-2 pandemic with high fatality. As vaccines are developed through various strategies, their immunogenic potential may drastically vary and thus pose several challenges in offering immune responses against the virus. Methods: In this study, we adopted an immunoinformatics-aided approach for developing a new multi-epitope vaccine construct (MEVC). In silico approach was taken for the identification of B-cell and T-cell epitopes in the Spike protein, for MEVC various cytotoxic T-lymphocyte, helper T-lymphocyte, and B-cell epitopes with the highest affinity for the respective HLA alleles were assembled and joined by linkers. Results: The computational data suggest that the MEVC is nontoxic, nonallergenic and thermostable and elicit both humoral and cell-mediated immune responses. Subsequently, the biological activity of MEVC was assessed by bioinformatic tools using the interaction between the vaccine candidate and the innate immune system receptors TLR3 and TLR4. The epitopes of the construct were analyzed with that of the strains belonging to various clades including the emerging variants having multiple unique mutations in S protein. Conclusions: Due to the advantageous features, the MEVC can be tested in vitro for more practical validation and the study offers immense scope for developing a potential vaccine candidate against SARS-CoV-2 in view of the public health emergency associated with COVID-19 disease caused by SARS-CoV-2.

Keywords: Epitopes, peptide antigen, prophylaxis, SARS-CoV-2, spike protein, vaccine

INTRODUCTION

There is an urgent need to craft vaccines for SARS-CoV-2 for reinforcing immune defense against the virus including the new variants.1-7 Development of multi-epitope vaccine constructs (MEVCs) has the advantages such as speed, safety, chemical stability, and selective activation of immune responses. The design of a multi-epitope vaccine depends on the identification and assembly of B- and T-cell epitopes that are capable of stimulating the humoral and cell-mediated immune.7-14

In this the current study, using immunoinformatics tools11,15-21 we predicted the cytotoxic T-lymphocyte (CTL), helper T-lymphocyte (HTL), and B-cell epitopes of spike protein from an isolate of our study and analyzed the conservancy and other immunological properties with respect to various Indian and global strains representing all clades including the new variant SARS-CoV-2 through immunoinformatic tools. We also investigated the population coverage of B- and T-cell epitopes from various countries affected by COVID-19. The interactions between the epitopes and their corresponding

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Address for correspondence: Dr. Satish Srinivas Kitambi, Institute for Healthcare Education and Translational Sciences, 10-2-311, Plot 187, Str 4, Cama Manor, West Marredpally, Secunderabad - 500 026, Telengana, India. E-mail: satish.kitambi@klife.info

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alleles were studied. Subsequently, the MEVC was designed and its biological activity was assessed by bioinformatic tools using the interaction between the vaccine candidate and the innate immune system receptors TLR3 and TLR4. We strongly believe that the outcome of the present report will support the development of a potential vaccine candidate against all SARS-CoV-2 variants.

**Methods**

**Sequencing and sequences retrieval**

Clinical samples were tested in King Institute of Preventive Medicine and Research, India by Real-time reverse transcription-polymerase chain reaction using TaqPath Multiplex Combo kit (Thermofisher). The RNA of SARS-CoV-2-positive samples was purified and sequenced. For the study, 41 full-length genome sequences of SARS-CoV-2 including the sequences representing different Variant of Concern (VOC) as well as Variant under Investigation (VUI) retrieved from GISAID were used and compared with the Wuhan, China (Wuhan hu-1) reference strain sequence (NC_045512.2) as well as GISAID reference strain (EPI_ISL_402124). Further, the mutations specific to the spike protein of various SARS-CoV-2 isolates were identified. Details of the structural and functional prediction of spike proteins including mutational analysis are shown in supplementary files which can be obtained by contacting the author directly.

**Designing of multi-epitope vaccine construct**

For constructing a multi-epitope vaccine construct, the selected HTL, CTL, and B-cell epitopes were joined by using GPGPG, AAY, EAAAK, and KK linkers, respectively. Four adjuvants namely, β-defensin, universal memory T-cell helper peptide (TpD), PADRE (Pan HLA-DR reactive epitope), sequence and an M-cell ligand were also added by using linkers into the vaccine construct. To enhance the immunogenicity, β defensin was added to the N terminal whereas in the C terminal, M-cell ligand was added which was followed by the addition of HHHHHH for facilitating purification of the vaccine. For the construction of a multi-epitope vaccine against SARS-CoV-2, the method of Chauhan et al. was adopted with the following criteria: They (a) should be promiscuous, (b) should have overlapping CTL and HTL epitopes, (c) immunogenicity, (d) population coverage, (e) high affinity toward HLA alleles, and (f) should not overlap with any human gene. Based on these criteria, the HTL and CTL epitopes were included in the final construct of the multi-epitope vaccine.

**Antigenicity, allergenicity, and physicochemical properties prediction**

The antigenicity of the vaccine was determined using the VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). The allergenicity of the vaccine was examined using AllerTOP v2.0 (http://www.ddg-pharmfac.net/AllerTOP/). The physicochemical characteristics of the vaccine were determined using the ProtParam tool of the ExPASy database server (http://web.expasy.org/protparam/).

**Structure prediction, validation, and docking with the receptor**

The secondary structure of the subunit vaccine construct was predicted using PSIPred 4.0 Protein Structure Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/), while the tertiary structure was predicted by GalaxyWeb server (http://galaxy.seoklab.org/tbm). The model of the vaccine construct with the best TMscore was validated by PROCHECK v. 3.5 (https://servicesn.mbi.ucl.edu/PROCHECK/) and ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) web servers. Vaccine-receptor docking was performed by the Cluspro v. 2 (https://cluspro.bu.edu/) protein-protein docking web server to determine the binding affinity of the vaccine with the TLR3 receptor (PDB ID: 2A0Z) and TLR4 receptor (PDB ID: 3FX1). To characterize the real-life immunogenic profiles and immune response of the multi-epitope vaccine, C-ImmSim server was utilized.

**Results**

**Sequence retrieval and prediction of physicochemical properties**

A S protein sequence of hCoV-19/India/CCMB_C17/2020 | EPI_ISL_458035 was selected for designing of epitopes for MEVC [Figure 1]. The Maximum Likelihood method was employed to draw phylogenetic tree from sequenced clinical samples and sequences from the GISAID database. For the prediction of T-cell and B-cell epitopes to design MEVC, the amino acid sequence of spike protein HCoV-19/India/CCMB_C17/2020 was selected. VaxiJen v2.0 was used to predict the antigenicity of the selected protein.

**T cell epitope prediction**

The CTL epitopes were predicted for all the selected proteins using the NetCTL 1.2 server and evaluated by the VaxiJen server. It was found that 41 epitopes among the 100 primarily selected T-cell epitopes were subjected to immunogenicity analysis, which revealed that 21 epitopes had the immunogenicity value > 0.00. From the shortlisted 21 epitopes, 10 of them showed 100% conservancy, non-toxic and non-allergen properties [Table 1]. The selected 10 T-cell epitopes were found to be recognized by the MHC class-I molecules. In this study, we chose IC50 values <100 nM (IC50 <100) for ensuring high affinity. For identifying HTL epitopes, a key player of the adaptive immune response, 14 epitopes were predicted using the IEDB MHC II server [Table 2] and subjected for their corresponding allele selection based on their affinity. Both HTL and CTL epitope structures were modeled using Pepfold-3 and their interaction with their respective HLA alleles were studied using PatchDock and FireDock.

**Linear/continuous B cell epitope prediction**

The prediction of B-cell epitopes was performed through the web server ABC pred [Table 3]. Identification of discontinuous/
conformational B cell epitopes was based on the 3D structure of the selected spike protein sequence. The 2D structures of

 spike protein predicted by the SOPMA tool, and 3D structure was predicted by the SWISS-MODEL server and refined by
3D Refine. PROCHECK was used to check the stereochemical quality of the structure. To check the potential errors of the protein 3D model, ProSA was used. A total of 6 discontinuous or conformational B-cell epitopes were predicted using the ElliPro tool of IEDB. Conformational epitopes and their individual residues, residue position, length, and the scores, whereas the positions of epitopes on 3D structures are displayed.

**Table 3: Features of linear or continuous b cell epitopes predicted from spike protein of SARS-CoV-2**

| Epitope      | Score | Start position | Antigenicity | Allergenicity | Toxicity | Conservancy (%) |
|--------------|-------|----------------|--------------|---------------|----------|-----------------|
| GVSVITPGTNNSQVA | 0.95  | 594            | 0.4651       | NA            | NT       | 100             |
| GWTAGAAAYVYGLQP  | 0.95  | 257            | 0.6210       | NA            | NT       | 100             |
| HRSYLTPGDSGGWTA  | 0.92  | 245            | 0.6017       | NA            | NT       | 100             |
| TVEKGIYQTSNFRVQP | 0.91  | 307            | 0.6733       | A             | NT       | 100             |
| GCLIGAEHVNNSYEC | 0.90  | 648            | 0.8480       | NA            | T        | 97              |
| LQSYGFQPTNGVYQP  | 0.90  | 492            | 0.5258       | NA            | NT       | 100             |

NT: Nontoxic, NA: Nonallergic, T: Toxic

**Mutation analyses of epitopes across various SARS-CoV-2 isolates and variants**

Totally 3156 sequences of spike protein along with 11 variants were analyzed for mutations with respect to the 36 selected CTL, HTL, and B-cell epitopes. Among the 36 epitopes, 20 epitopes were highly conserved suggesting there are no mutations in them. Totally, 29 distinct substitution mutations were identified in the remaining epitopes of isolates of VOC and VUI. These results suggested that all the epitopes selected for the MEVC had high degree of conservancy among all the variant strains belonging to various distinct clades.

**Designing of multi-epitope vaccine construct**

The antigenic 14 HTL and 10 CTL epitopes possessing the highest affinity for the respective HLA alleles and four B-cell epitopes that displayed non-allergenic, nontoxic, and immunogenic features were selected for incorporation into the MEVC. The adjuvant β-defensin was coupled at the N terminal by EAAAK linker with B cell epitope and subsequently, AAY, GPQPG, and KK linkers were used to couple B-cell epitopes, CTL epitopes, and B HTL epitopes, respectively. Adjuvants like Universal memory T-cell helper peptide (TpD), PADRE (Pan HLA-DR reactive epitope) and M cell ligand were coupled by using EAAAK linkers into the vaccine construct. HHHHHH was coupled at the C terminal by EAAAK linker for the easy purification of the vaccine [Figure 1]. The final MEVC was composed of 575 amino acid residues, which was then validated for antigenic, allergenic, and physiochemical properties.

**Physiochemical properties, antigenicity, and allergenicity of multi-epitope vaccine construct**

The physiochemical properties of the MEVC were calculated by the ProtParam tool [Table 4]. The total number of amino acids in the MEVC was 575 with the molecular weight of 61480.71. The total number of positively charged residues (Arg + Lys) and negatively charged residues (Asp + Glu) were 67 and 21 respectively. The theoretical isoelectric point (pI) was calculated as 9.92. Grand Average of Hydropathicity (GRAVY) was 0.001. Upon analyzing the vaccine construct sequence in the VaxiJen server, the constructed vaccine was found to be antigenic in nature with an overall prediction score of 0.6077. In summary, the constructed epitope was observed to be stable, soluble, antigenic, non-allergic, and nontoxic.

**Secondary and tertiary structure prediction and validation**

The secondary structure of the construct was analyzed by using the SOPMA server that revealed the presence of −29.57% α-helix, −24.35% β-sheet, −37.91% coils and −8.17% β-turns in the vaccine construct [Figure 2]. The tertiary structure of the MEVC was predicted by the Galaxy WEB server and refined by Galaxy Refine [Figure 3a]. For the selected best model, the GDT-HA, RMSD, and MolProbity scores were −0.9962, −0.243, and 2.279, respectively. PROCHECK was used to check the stereochemical quality and Ramachandran plot analysis of the modeled structure revealed the presence of 89.8% residues in the most favored regions and 8.2% in the additionally allowed regions, 1.2% in the generously allowed region, and 0.8% in the disallowed region [Figure 3b]. To check the potential errors of the protein 3D model, ProSA was used, and it predicted the negative Z-score of −3.65 suggesting the good quality of the model [Figure 3c]. These results substantiated the quality of the predicted model.

**Docking of multi-epitope vaccine construct with receptors**

The 3D structures of human TLR3 and TLR4 were retrieved from protein data bank (PDB ID: 2A0Z and 3FX1). Molecular docking analysis was performed using the ClusPro v. 2 protein-protein docking server. Cluspro v. 2 predicted 29 models each of vaccine receptor TLR3 complex and TLR4 complex with their corresponding cluster scores. Among these models, model number 1 (cluster 0) in TLR3 and TLR4 complex were selected as the best-docked complex with the lowest energy score of −1274.5 with 29 members (TLR3) and lowest energy score of −1329.1 with 29 members (TLR4). This signifies potential molecular interaction between predicted vaccine construct with TLR3 and TLR4 receptors.

**Population coverage of multi-epitope vaccine construct**

Nine countries showed 100% population coverage while ≥99% of the population was covered in 35 countries whereas ≥95% was covered by 23 countries. In 10 countries, ≥90% of the population was covered. Hence, the vaccine construct showed 90%–100% population coverage in 77 countries and 99.9% population coverage throughout the world.
Immune simulations of vaccine construct

C-ImmSim simulator was used to analyze the immune response produced by the final vaccine construct. The total simulation is focused on three events: (1) B-cell epitopes binding, (2) HLA Class I and II epitopes binding, and (3) TCR binding, which HLA-peptide complex interaction should be presented. The cumulative results of immune responses after three times antigen exposure revealed that the primary immune response against the antigenic fragments was elevated and it was indicated by the gradual increase of IgM level after each antigen exposure. Similarly, the secondary response was characterized by the adequate generation of IgM + IgG more than IgM. An increased level of IgG1 + IgG2 and IgG1 was also observed. On the subsequent exposure of the vaccine, a decrease in the level of antigens was observed indicating the development of immunogenic response in the form of immune memory. The elevated levels of all circulating immunoglobulins indicate the accuracy of the relevant clonal proliferation of the B-cell and T-cell population. Furthermore, an increase in the B-cell population was characterized by an increase in the expression

Table 4: Physiochemical properties of the multiepitope vaccine construct

| Features                              | Value    |
|---------------------------------------|----------|
| Number of amino acids                 | 575      |
| Molecular weight                      | 61480.71 |
| Theoretical PI                        | 9.92     |
| Total number of negatively charged residues (asp + glu) | 21       |
| Total number of positively charged residues (arg + lys) | 67       |
| Total number of atoms                 | 8752     |
| Instability index                     | 26.87    |
| Aliphatic index                       | 84.07    |
| GRAVY                                 | 0.001    |

GRAVY: Grand average of hydrophaticity, PI: Isoelectric point
of immunoglobulins, which resulted in a decrease in the concentration of the antigen. Besides, there was a consistent rise in Th (helper) and Tc (cytotoxic) cell population with memory development. Total NK cells, dendritic cells, and macrophages were also increased. It was also observed that the production of IFN-gamma was stimulated after immunization. These results revealed that the MEVC proposed in this study could generate a strong immune response, and immunity increases even on subsequent repeated exposure.

**DISCUSSION**

For developing efficient epitope-based peptide vaccine, surface glycoprotein of virus is considered as the major focus by vaccine design platforms.[22-26] In the present study, we attempted reverse vaccinology approach for designing of a multi-epitope vaccine based on the Spike (S) protein of SARS-CoV-2 that may efficiently elicit humoral and cellular mediated immune responses against the viral infection.[27] Substitution mutations in spike protein sequences of SARS-CoV-2 isolates from COVID-19 positive clinical samples of Tamilnadu, India and 20 other strains representing various clades and emerging VOC and VUI strains were included in the analysis in comparison with the reference sequence of Wuhan strains and a phylogenetic tree was constructed employing MEGA. Among the 41 sequences, a sequence of an isolate was selected to predict various B-cell and T-cell epitopes against SARS-CoV-2.

The retrieved structural protein and its antigenicity score suggest that the spike protein is the most potent protein to generate immune response. Both T- and B-cell epitopes were predicted using immunoinformatics tools. MHC class I binding peptides generally have 8-11 amino acids while MHC class II binding peptides are typically 12-25 amino acids long. The study results on T cell epitope prediction and analyses based on features such as antigenicity, allergenicity, immunogenic conserved, and toxicity suggested that the selected T-cell epitopes had high scores for these features. The lower percentile rank and lower IC50 (<100 nM for Class I and <1000 nM for Class II) values for alleles of T cell epitopes met the criteria to be strong binders supporting the allele selection for population coverage. Further, the B-cell epitopes were divided into two main categories such as continuous or linear B-cell epitope and discontinuous or conformational B-cell epitope. The predicted linear B-cell epitopes with higher cut-off values (0.9 and above) were analyzed for antigenicity, allergenicity, toxicity, and conservancy and the best scoring epitopes were selected for MEVC. The secondary and tertiary structures of the spike protein sequence were predicted and validated to identify conformational or discontinuous B-cell epitopes. Totally, 6 epitopes were predicted with high score.

Vaccine construct should be antigenic, non-allergenic, and nontoxic to make it a potent vaccine candidate against SARS-CoV-2. Hence, 36 epitopes such as 10 CTL, 14 HTL and 12 B-cell epitopes were chosen for MEVC design and the epitopes were selected for the study based on the conservancy, antigenicity, nontoxicity, and non-allergenicity features. It is noteworthy that the 36 epitopes selected for the MEVC had high degree of conservancy with respect to these epitopes of all variants of distinct clades including the recently emerged VOC and VUI strains. The analyses of mutations in the epitopes of MEVC against VOC and VUI strains indicate that the MEVC can confer immunity against these variants due to the insignificant number of mutations in the epitopes.

These epitopes were linked by adjuvants and linker molecules. Four different adjuvants such as β-defensin, TpD, PADRE and M-cell ligand were added to the MEVC to enhance the innate and adaptive immune responses besides aiding the transportation of MEVC through the intestinal membrane barrier. Analysis of the physicochemical properties of MEVC indicated that this protein could have stability in several temperatures. This construct showed high antigenicity and it was nonallergic and non-toxic. In this study, the MEVC was docked with TLR3 and TLR4. The molecular interaction of vaccines with TLR3 and TLR4 through docking analysis suggested that the constructed vaccine possessed a significant affinity toward the toll-like receptors to recognize molecular patterns of the pathogen to initiate the immune response. The adjuvant β-defensin in the present MEVC acts as a TLR agonist which can interact with multiple TLRs to stimulate both innate and adaptive immunity against viral infections. Thus, the MEVC with the β-defensin adjuvant is capable of generating an effective immune response against SARS-CoV-2.

Based on the presence of epitopes for the HLA alleles, the population coverage of the vaccine showed 99.9% of the world population. The consistent increase of high level of IFN gamma supported the activation of humoral immunity. Further, CTL, HTL, and B-cell epitopes incorporated in the MEVC revealed that all these epitopes were highly conserved.

Though the study analyses were made based on the spike protein of SARS-CoV-2, an earlier study reported the analyses of CTL, HTL and B-cell epitopes from 3 different proteins of the virus.[28] The spike protein is a key target for the development of vaccines, therapeutic antibodies, and diagnostics for coronavirus, and hence, it was chosen for the study. The inclusion of more potential epitopes from other proteins in the MEVC may suffer the limitation of the complexity of the construct besides the challenges associated with the synthesis. A recent study focused on the single protein (spike protein) to generate multiple epitopes such as 13 for MHC I and 3 for MHC II epitopes;[27] but this study has the limitation of not considering the B-cell epitopes. Another recent study reported the design of subunit vaccines against SARS-CoV-2 that used only CTL epitopes without considering the significance of B-cell or HTL epitopes.[29] Some studies have used other proteins of the virus and restrict the analyses to one of these three epitopes.[30]
**Conclusions**

The present study has an advantage in providing a potential MVEC as it contains all three types of epitopes such as CTL, HTL, and B-cell epitopes. This *in silico* study is an attempt to describe the potential immunogenic target over the structural proteins and to propose a novel MVEC, for providing new rays of hope in the initial phase of vaccine development and subsequent experimental validation to confer protection against SARS-CoV-2 infection.

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**Research quality and ethics statement**

This study was determined not to require IEC approval. The authors followed applicable EQUATOR Network ("http://www.equator-network.org") guidelines during the conduct of this research project.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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