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Original Article

In Silico Design of a Multimeric Polytope as a Highly Immunogenic DNA Vaccine Against Human Cytomegalovirus

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Abstract

Human cytomegalovirus is one of the most common pathogenic viruses all over the world. In congenital infection leads to neurologic severe disorders and even death of fetus and in individuals with immunosuppression may also cause severe clinical symptoms. Multiple evidence indicate that among several strategies, epitope-based vaccine (EVs) that can induce both humoral and cellular immunity responses, are the most important and have numerous potential profits. In this study, we select the viral surface glycoprotein B and phosphoprotein 65 and 150 with the highest antigenic and immunogenic properties, that have the most important role in induce cellular and humoral immune responses. Bioinformatics tools, as a standard and developed approaches use for epitope mapping. Epitope discovery greatly accelerate by in silico prediction methods with in vitro and in vivo verification. Bioinformatics methods and epitopes identification algorithms were used in order to selection of cytomegalovirus immunodominant epitopes, detection of each epitope antigenicity and design chimeric gene construct. The chimeric protein showed high antigenicity in vaxiJen analysis. Also further immunoinformatic analyses in order to predict the discontinuous and continuous B and T cell epitopes and MHC binding peptides affinity were used. The study results show that protein structures were suitable. Therefore it can be expected that construct is proper subject for practical experiments and stimulus for humoral and cellular immune responses.

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Introduction

Human cytomegalovirus (HCMV), a ubiquitous betaherpesvirus (type 5), with a double-stranded DNA genome of about 235 kb encoding 165 genes, is the largest member of human herpesvirus family [1]. Depending on socioeconomic situations, Its propagation is nearly 80% in the USA and Europe and 100% in Asia and Africa [2]. HCMV can be transmitted through breast feeding, saliva, placental transfer, blood transfusion , sexual contact, hematopoietic stem cell transplantation (HSCT), and solid-organ transplantation (SOT) [3]. Although HCMV create spermanent latency and sporadic reactivation in the host, primary HCMV infection doesn't cause clinical illness in immunocompetent people [1]. Primary and reactivated HCV infection in healthy individuals is mostly asymptomatic, although symptomatic diseases, such as infectious splenomegaly, mononucleosis, and a mild hepatitis can be observed in some individuals [4, 5]. It can lead to severe disease, and even death, among immunocompromised populations, such as the fetus and infants with an undeveloped immune system, patients infected with human immunodeficiency virus (HIV), and solid organ and hematopoetic stem cell transplant recipients [6, 7]. A recent study has shown a possible role of HCMV in the expansion of different inflammatory diseases, for instance, autoimmune diseases, vascular diseases, hepatitis, gastrointestinal diseases, pneumonia, and atherosclerosis [8], it can also increase the risk of graft failure, illness, and even death in hematopoietic stem cell and solid-organ transplant recipients and is the major cause of morbidity and mortality in congenitally CMV infected infants [9-11]. Congenital infection in infants leads to important neurologic disorders, such as cerebral palsy, seizure disorders, mental retardation, developmental delay, microcephaly with intracranial calcifications, chorioretinitis, skin lesions, such as petechiae and purpura, visceral organomegaly, and permanent disabilities, such as serious hearing and vision loss and cognitive impairment [12-14]. Therefore, HCMV infection is an important public-health problem. Although there are some antiviral drugs for the treatment or prevention of CMV associated diseases that reduce the risk of CMV infection in transplant recipients, however, their cure rates are not acceptable and these strategies have not had an important effect on the reduction of congenital HCMV infection rate [7, 10]. Also there are some other problems, such as severe side effects, drug resistancy of HCMV mutated strains, drug toxicity, the expense of antiviral treatment, and the development of viremia in patients [9, 15]. Thus an efficient vaccine to protect against HCMV-associated disease and offers a reduced need for antiviral therapy, is the most effective prophylactic strategy against HCMV

infection and is an essential public health priority. Several approaches have been designed to develop vaccines against HCMV, but unfortunately, more than 30 years of research have not yielded an efficient vaccine [6, 7, 16]. Although the innate immune system has an important role in the protection against HCMV disease and infection, adaptive immunity (both humoral and cellular) is essential for the prevention of the infection [17, 18]. Thus, an optimal HCMV vaccine must induce both humoral and cellular immune responses [10]. Among several vaccine strategies, epitope-based vaccine (EVs) that can induce protective humoral and/or cellular immune responses have numerous potential profits over traditional vaccines, such assafety, easy construction, chemical simplicity, determined formula, accurate control over the immune response activation and the capability of focusing on the most significant antigenic segments. Since experimental screening is time consuming and expensive, bioinformatic and in silico techniques that facilitate epitope mapping on protein antigens are preferred for EV analysis [19].

In recent years, bioinformatics and computational algorithms are widely used as a standard and developed approaches for epitope mapping. Epitope discovery is greatly (nearly 10–20-fold) accelerated by the in silico prediction methods with in vitro and in vivo verification [20, 21]. The accelerating progress of bioinformatic applications in immunology has created a novel research field, named immunoinformatics that have significant applications in vaccine research, including the possibility of the determination of binding affinity of antigenic peptides, the characterization of virulence genes and surface-associated proteins [22], the determination of the set of immunogenic epitopes (immunome), theprediction of protein–protein (antigen–antibody) interactions, codon optimization, the prediction of protein structure and functions [23, 24], the capability of decreasing of problems and time involved in the screening of potential epitopes, and the capability offinding the conserved epitopes [25].

In the present study, we selected the conserved regions of the important viral antigens and used bioinformatic tools to choice the best epitopes. We designed a novel recombinant gene construct containing multi antigenic epitopes that provides a suitable and safe polytope DNA candidate vaccine against HCMV.

Materials and Methods

Study design

The present study consists of two parts. In the first part, analysis and database searching were performed to find the conserved and specific regions of HCMV genome in order to select immunogenic and find a proper region that is expressed in several species of CMV. The second part consists of various analyses of the selected gene, such as epitope mapping, prediction of secondary and tertiary structures, antigenicity determination, and B and T-cell epitope prediction.

Sequence availability and database searching

In this study information on T-cell Epitope sequences were obtained from related studies [26-28]. The selected sequences of the antibody-binding sites were obtained from the Uniprot Database at http://www.uniprot.org (Protein ID (Uniprot): P06473) and NCBI at http://www.ncbi.nlm.nih.gov/(GenBank:ACL51135.1GI:219879600). The sequences submitted to the Basic Local Alignment Search Tool (BLAST) to ensure that the selected sequences were conserved.

Primary analysis and chimeric construction design

The five selected segments were fused together for designing a synthetic chimeric construction. For determination of various physicochemical parameters, such as amino acid composition, molecular weight, theoretical pI, instability index, total number of negatively and positively charged residues, and half-life and aliphatic index estimation, the “protparam” [29] online software at http://expasy.org/tools/protparam.html were exploited [21].

Other features of the resulted recombinant protein, such as flexibility [30], hydrophilicity [31], number and position of the turns [32], polarity, exposed surface and antigenic propensity [33, 34] were calculated at http://web.expasy.org/protscale/. In order to estimate the antigenicity of the chimeric protein and each sequence, VaxiJen software [35] at http://www.ddg pharmfac.net/vaxijen/VaxiJen/VaxiJen.html was used [21, 36].

Secondary Structures Prediction

The evaluation of the secondary structure of the chimeric protein was performed by GOR online software [37, 38] at http://npsa-pbil.ibcp.fr/cgi-bin/secpred_gor4.pl [21,39]. Predict Protein server [40] was exploited for sequence analysis and also for protein structural and functional evaluation including low-complexity regions, transmembrane helices, regions lacking regular structure, coiled-coil regions, solvent accessibility, secondary structure,and disulfide-bonds [36].

Tertiary Structure Prediction

In order to identify the chimeric protein 3D models, I-TASSER online software [41] was used [42]. In order to visualize 3D protein structures, Accelrys Discovery Studio 1.7 was used.

Gene optimization

For the optimization of “AD1-T2-T3-T1-AD2” multimeric construct’s codons, “GenScript” service (http://www.genscript.com/gene_synthesis.html) was used [43].

mRNA secondary structure prediction

For mRNA secondary structure analysis, the “Mfold” server at http://www.bioinfo.rpi.edu/ applications/mfold was used [42, 44].

Immunoinformatic analysis

Predicting the discontinuous and continuous B-cell epitopes

ABCpred (http://www.imtech.res.in/raghava/abcpred/), BcePred (http://www.imtech.res.in/raghava/bcepred/) [45], BepiPred (www.cbs.dtu.dk/services/BepiPred/) [46], ElliPro(http://tools.immuneepitope.org/tools/ElliPro/iedb_input) [47] and IEDB (http://tools.immuneepitope.org/tools/bcell/iedb_input) [48] online software were usedfor linear (continuous) B cell epitopes analysis. In addition, the prediction of the conformational (discontinuous) B cell epitopes was performed using Ellipro and CBTOPE (http://www.imtech.res.in/raghava/cbtope/) online servers [45].

Prediction of T-cell epitopes and MHC binding peptides affinity

Three predictive algorithms:
SYFPEITHI (http://www.syfpeithi.de), BIMAS (http://www-bimas.cit.nih.gov/index.shtml), and CTLPred (http://www.imtech.res.in/raghava/ctlpred/index) were used to predict the CTL epitopes [27, 42].

Results

Sequence availability and database searching

Immunological studies that determine the immunologically important HCMV antigens, have demonstrated that the tegument phosphoprotein pp65 is the most dominant target for cellular immune response stimulation against HCMV [49]. Another recognized antigen of HCMV, that can induce specific CTLs responses, is pp 150 [27]. Similarity searches have also shown that the glycoprotein B (gB) is a principal target for neutralizing antibodies against HCMV [9, 50].

In this study, we selected a pp65-derived epitope (RQY16-mer), defined by specific features, such as the capability of inducing immune reactions in both $CD4^+$ and $CD8^+$ T cells, even in pharmacologically immunosuppressive individuals, high immunogenicity, the ability of stimulating the production of TNF-α, INF-γ and proliferating $CD4^+$ T cells [26]. Other information on T-cell epitope sequences was obtained from the references [27] and [28] involving the recognition of epitopes and efficient sites (Table 1) [26-28, 63, 64].

Table1. HLA-restricted T-cell epitopes of HCMV included in the chimeric construct

T-cell Epitope sequences	HLA	HCMV antigens	ORF Names
RQYDPVAALFFFDIDL		$pp65_{340-355}$	UL83
ARNLVPMVATVQGQN -LKYQEFFW	A2	$pp65_{493-515}$	UL83
WPRERAWAL	B7/B8	$pp150_{641-649}$	UL32

Conformational and linear binding sites on the glycoprotein B molecule have been mapped by bioinformatic analysis. Linear binding sites have two conservative linear epitopes on SU segment and also two linear antibody sites on TM segment [51]. Accordingly, we chose two regions that stimulate the production of antibodies against HCMVefficiently (Table 2) [65-68].

The selected sequences of the antigen glycoprotein gB were obtained from the Uniprot Database at http:// www.uniprot.org (Protein ID (Uniprot): P06473) and NCBI at http://www.ncbi.nlm.nih.gov (GenBank: ACL51135.1GI:219879600). The sequences submitted to the Basic Local Alignment Search Tool (BLAST) and it was seen that the selected sequences were extremely conserved between various strains of CMV.

Primary analysis and chimeric construction design

The five selected segments were fused together for designing of a synthetic chimeric construction. For correct translation and overexpression of mRNA in the eukaryotic host up to 10 fold, the Kozak sequence [52] was inserted before initiation codon. In addition, a 6-histag was inserted to its C-terminus of the recombinant protein for confirming and, also, the easy purification of the chimeric protein.

Table 2. Major antibody binding site in gB HCMV included in the chimeric construct.

Antibody-binding site	HCMV antigens	ORF Names
AD2	gB_{27-86}	UL55
AD1	$gB_{540-645}$	UL55

Since the KDEL sequence in the carboxy-terminal of the protein is necessary for the accumulationof the chimeric protein inside the ER, the proper sequence was added at the 3' end of the synthetic chimeric gene [20]. The arrangement of the fragmentsis shown in figure 1.

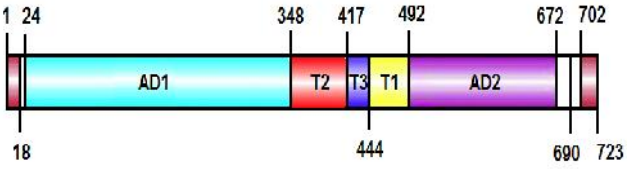

Figure 1. Schematic representation of the chimeric construct, T2: $pp65_{493-515}$, T3:pp $150_{641-649}$, T1: $pp65_{340-355}$.

Various physicochemical parameters of the chimeric protein construct were computed by "protparam" server. The molecular weight of the recombinant protein was calculated as 25.5996 kDa, theoretical isoelectric pH is approximately 6.44,the instability index was computed to be 31.32 (and this shows that the protein is classified as a stable protein in a eukaryotic cell), aliphatic index was 77.17 and the total number of negatively (Asp + Glu) and positively (Arg + Lys) charged residues were respectively 22 and 19.

The amino acid composition was also calculated; some amino acids such as serin, valine, leucine, threonine, alanine, and asparagine were found to be more abundant (48.1%) in the protein than other amino acids. In addition, the antigenicity of the whole chimeric protein and each sequence was estimated (Table 3). Among the all forms of epitopes arrangement, "AD1-T2-T3-T1-AD2" that has the highest antigenic index (0.64) and the best secondary and tertiary structures, was selected.

Table 3 . The antigenicity score of each sequence according to vaxiJen server

Antibody-binding site	VaxiJen scores	T-cell Epitope sequences	VaxiJen scores
AD2 (gB_{27-86})	0.6110	$pp65_{340-355}$	0.8969
AD1($gB_{540-645}$)	0.4728	$pp65_{493-515}$	1.0890
		$pp150_{641-649}$	1.5113

Secondary Structures Prediction

The secondary structure of the chimeric protein was predicted by two prediction methods. The results indicate that alpha helix (25.00%), extended strand (15.28%) and random coil (59.72%) are structural constituents of the chimeric protein. The schematic results of protein secondary structure prediction are depicted in Figure 2.

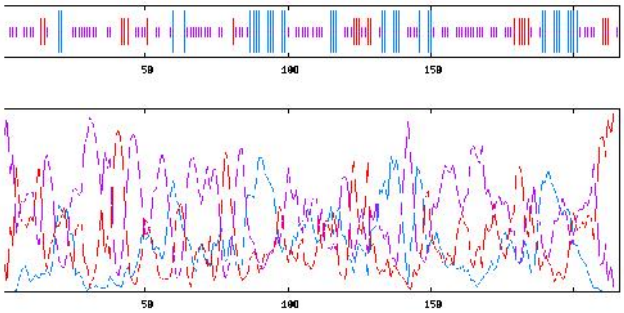

Figure 2. Graphical results of thepredicted secondary structures of the chimeric proteins. Extended strand: purple, Coil: red, Helix:blue.

Tertiary Structure Prediction

I-TASSER server http://zhanglab.ccmb.med.umich.edu/I-TASSER/, developed by Michigan University, were used for the prediction of the protein structure. Model 1 was chosen based on its confidence score (-1.60). The confidence scoreis useful for the determination of the quality of the predicted models and is typically in the range of [−5 to 2]. AC-score of higher value signifies a high confidence (high reliable) model. Also, the expected template modeling (TM-score) of the model was estimated to be 0.52±0.15 and the expected root-mean-square deviation (RMSD) was 9.0 ± 4.6 Å. The prediction of 3D models of the chimeric protein by I-TASSER are estimated based on combining the multiple threading models, structural refinement, and ab initio modeling. Tertiary structures of the chimeric protein construct are illustrated in Figure 3.

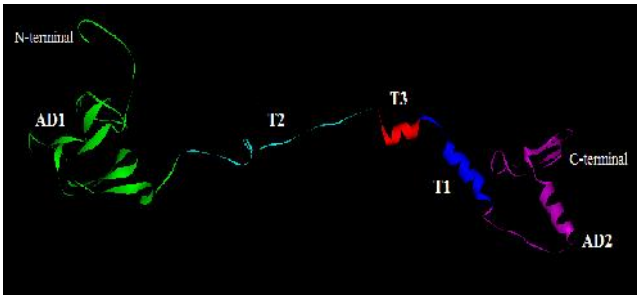

Figure 3. Three dimensional structures of the (AD1-T2-T3-T1-AD2) construct showing the different domains and two terminal regions.

Gene optimization

"GenScript's OptimumGene™" service is a gene optimization technology that can modify both recombinant and naturally gene sequences to achieve the highest conceivable levels of expression in any expression system. The "OptimumGene™" algorithm considers different critical factors involved in various stages of protein expression, such as frequency of optimal codons (FOP), GC content, mRNA secondary structure, codons adaptability, various cis-acting elements in transcription and translation, restriction sites, and removing of repeated sequences.

The optimized chimeric gene had not any rare codon and its codon adaptation index (CAI) was improved to 0.94 (Fig. 4a). Codon Adaptation Index (CAI) of a gene demonstrates the relative usage of codons of the desired gene in comparison to the codon usage of highly expressed genes. The percentage distribution of codons having a frequency of 91–100 in the optimized gene sequence was significantly improved to 82% (Fig. 4b).

The ideal percentage range of GC content of a gene is between 30-70%. To improve the efficiency of transcription and translation, and, also, to increase the stability of the related mRNA, any peaks outside of this range were removed. The average GC content of the optimized sequence is 58.98.

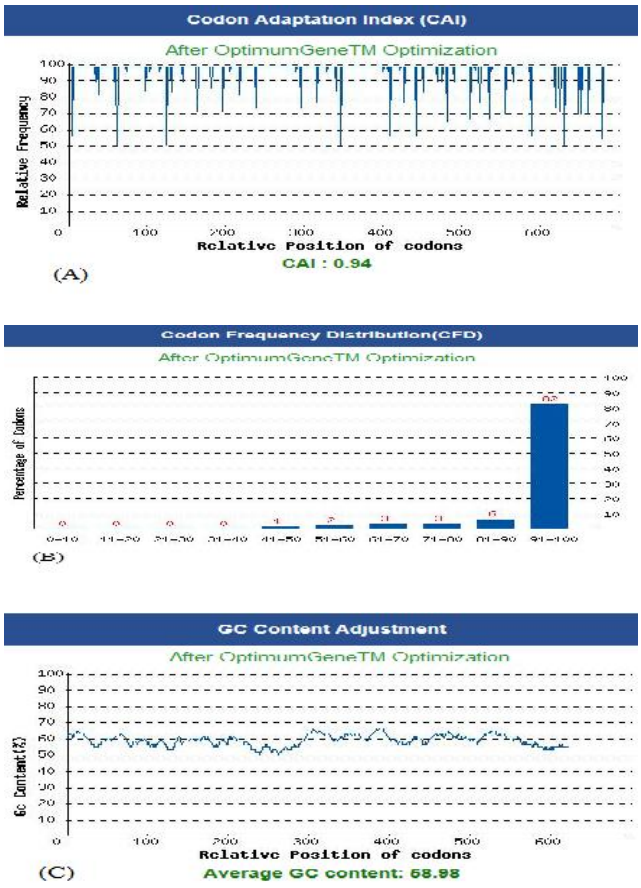

Figure 4. Codon usage bias adjustment of the optimized gene for expression in human. (**a**); codon adaptation index (0.94) indicate high gene expression levels. (**b**); percentage distribution of codons. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. (**c**); GC content adjustment; the ideal percentage range of GC content is 30-70%.

The Stem-Loop structures, that have an influence on ribosomal binding and, also, the stability of mRNA, were broken. Furthermore, negative cis-acting elements, negative repeat elements, and antiviral motifs, that all can decrease the efficiency of the translation has been removed or successfully modified. Filtered restriction enzyme sites are related to BamHI, BglII, EcoRI, HindIII, NdeI, SalI, XbaI, XhoI restriction sites. We added start codon in the start position of the genes and stop codon after C-terminal 6His-tag and the KDEL sequence. Also the 5'GCCACC sequence was inserted before the initiation codon. Alanine was the second codon following the initial methionine, encoded by the codon GCA, Finally, the necessary restriction sites (BglII, EcoRI, and XbaI at the N-terminal and HindIII, XhoI, and SalI at the C-terminal) were considered for cloning purposes in eukaryotic vectors.

mRNA secondary structure prediction

Prediction of mRNA secondary structure of the chimeric gene using mfold algorithm showed minimum free energy for all 27 predicted structures. ΔG of the best predicted structure for the optimized construct was calculated to be -224.60 kcal/mol. The data showed that the mRNA was stable enough for efficient translation in a new host. Predicted structure has no long stable hairpin and pseudoknot at the 5' site of mRNA.

Immunoinformatic analysis

Predicting the continuous and discontinuous B-cell epitopes

Those flexible epitopes that could interact easily with antibodies, always located on the surface of the protein. Many factors including hydrophilicity, exterior accessibility, polarity, flexibility, secondary structure, exposed surfaces, and antigenicity (Fig. 5) are important for the prediction of the recombinant protein epitopes. For this purpose, the BcePred and IEDB programs were employed for the determination of the continuous B cell epitopes (Table 4).

Figure 5. Important properties of B cell epitope prediction with IEDB. Hydrophilicity, flexibility, surface accessibility, beta-turn plots. Green regions under thethreshold color denotes unfavorable regions related to the properties of interest. Yellow colors are above the threshold sharing higher scores. Horizontal red line is the threshold.

The continuous and discontinuous B-cell epitopes were also predicted using Ellipro software, that its prediction is based on the 3D structures. All these approaches were performed at www.immuneepitope.org. The analyzing of the 3D structure indicated 8 linear B-cell epitopes (Table 5) and four sets of conformational B-cell epitopes containing 11 (Score: 0.890), 52 (Score: 0.720), 17 (Score: 0.687)53 and 24 (0.653) residues. The scores are listed in Table 6. In addition, prediction of discontinuous B-cell epitopes, based on the primary sequence, was performed by CBTOPE online server [53] (Table 7). ABCpred results showed 20 linear 16-mers B-cell epitopes, ranking based on scores that were belongs to our selected region. Also B cell epitopes were also predicted by BepiPred. The application of this method to a large number of proteins allowed an accuracy of 75% with a sensitivity of 0.49 and a specificity of 0.75. The overlapped parts of the two software's results were considered as potential B cell epitopes (Table 8).

Prediction of T-cell epitopes and MHC binding peptide affinity

T-cell epitopes prediction and MHC class I-binding peptides affinity of the chimeric protein was performed by using the SYFPEITHI [54] and the BIMAS [55] servers.

SYFPEITHI server determines the ligation strength to a defined MHC type for a peptide sequence, and in order to predict CTL epitopes, the possibility of the processing and presenting peptide is given. The SYFPEITHI scores are shown in Table 9 [42]. BIMAS (Bioinformatics and Molecular Analysis Section) server evaluated CTL epitopes based on a predicted half-time of dissociation to 46 HLA class I supertypes (Table 10). Epitopes that scored higher than 24 in the SYFPEITHI predictive algorithm and, also, peptides that ranked greater than 100 from the BIMAS server predictions are considered to be immunogenic epitopes [27].

Discussion

Cytomegalovirus infections and associated diseases have been an important health problem of the societies. Among many strategies, vaccination, specially, polytope DNA vaccine is a highly efficient option for HCMV infections control [1]. A large number of studies have shown that the cellular immune response to HCMV infection includes both MHC class I and II (restricted $CD8^+$ and $CD4^+$ T cell lymphocyte). Antigens as a candidate for vaccination, must be highly immunogenic, safe, and conserved that can induce immunity responses against wide spectrum of CMV strains. In order to induce CTL immune responses, most emphasis has focused on the tegument phosphoprotein 65 (pp65) as an immunodominant target. In addition, the pp150 protein (also known as ppUL32) has been recognized as a major target for HCMV-specific T-cell responses [14, 49].

The most fully characterized immunodominant antigen for inducing neutralizing antibodies production is surface glycoprotein B (gB, also known as gpUL55) [10]. Therefore, based on the previous studies, we choose three epitopes of pp65 and pp150 with high antigenicity scores that can induce cellular responses through a wide variety of HLA class I and II, and a region of gB for elicitation of antibody responses.

Table 4. Prediction of linear B-cell epitopes in recombinant protein based on different parameters by BcePred and IEDB software.

Prediction parameters	Epitope positions
Hydrophilicity	30-36, 55-62, 66-74, 105-111, 157-181, 194-200
Flexibility	27-33, 159-179
Accessibility	25-40, 48-54, 56-62, 65-75, 84-90, 120-147, 159-186 194-210
Exposed Surface	25-31, 121-128
Polarity	25-36, 65-75, 130-141, 190-201
Antigenic Propensity	5-24, 33-45, 49-56, 71-85, 88-94, 101-107, 109-124, 139-163, 162-172, 179-196

Table 5. Predicted linear B-cells epitopes based on the 3D structure of construct.

No.	Start position	End position	peptide	Number of residues	score
1	1	11	FMGDVLGLASC	11	0.890
2	69	76	EECQLPSL	8	0.775
3	42	52	FNFANSSYVQY	11	0.764
4	155	198	STSHATSSTHNGSHTSRTTSAQTRS-VYSQHVTSSEAVSHRANET	44	0.748
5	210	214	VGVNT	5	0.612
6	86	98	YEYVDYLFKRMID	13	0.574
7	14	18	INQTS	5	0.527
8	100	110	SSISTVDARNL	11	0.512

Table 6. Discontinuous B-cell epitopes predicted based on the 3D structure.

score	Number of residues	residues
0.890	11	F1, M2, G3, D4, V5, L6, G7, L8, A9, S10, C11
0.720	52	S155, S157, H158, A159, T160, Q:S161, S162, T163, H164, N165, G166, S167, H168, T169, S170, R171, T172, T173, S174, A175, Q176, T177, R178, S179, V180, Y181, S182, Q183, H184, V185, T186, S187, S188, E189, A190, V191, S192, H193, R194, A195, N196, E197, T198, N201, T202, D208, V210, G211, V212, N213, T214
0.687	17	I14, N15, Q16, T17, S18, I41, F42, N43, F44, A45, N46, S47, S48, Y49, V50, Q51, Y52
0.653	24	L22, R23, D24, P31, G32, R33, C34, E69, E70, C71, Q72, L73, P74, S75, L76, E87, Y88, V89, D90, Y91, L92, F93, K94, R95

The basic strategy in the present research, which was the design and construction of a polytope DNA vaccine, not only increased the immunogenicity of the chimeric antigen, but also facilitated the induction of humoral and cellular protective responses. For these goals, various bioinformatics methods and computational algorithms were used for prediction of T and B-cell epitopes and to accelerate the procedure of the research by selecting just those peptides that could imitate immunogenic antigens with excellent immunogenicity, instead of the complete protein, therefore, saving resources and time.

Table 7. Conformational B-cell epitopes from full length proteins according to CBTOPE server. CBTOPE has been developed for predicting B-cell epitopesof a protein from its amino acid sequence.

Amino acid	position	Probability scale	Amino acid	position	Probability scale
VKVL	21-4	4	N	61	4
RD	25-6	5	NHR	67-9	4
MN	27-8	6	T	70	5
V	29	5	E	71	6
K	30	7	EC	72-3	7
E	31	6	Q	74	6
SP	32-3	8	L	75	4
GR	34-5	7	P	76	7
C	36	8	S	77	7
YS	37-8	5	VA	116-17	4
P	40	6	A	137	4
VVI	41-3	5	SH	159-60	4
FN	44-5	4	AVS	192-4	4
ANSS	47-50	4	IYNT	201-4	4
Y	51	5	L	206	4
VQYG	52-5	4	NT	215-16	4

Table 8. Potential linear B-cell epitopes. Overlapped results of BepiPred and ABCpred.

Rank	Sequence	Start position	Score
1	HVTSSEAVSHRANETI	186	0.95
2	TTSAQTRSVYSQHVTS	174	0.92
3	GRCYSRPVVIFNFANS	34	0.89
4	KVLRDMNVKESPGRCY	22	0.86
4	HATSSTHNGSHTSRTT	160	0.86
5	RERAWALRQYDPVAAL	134	0.82
6	YQEFFWWPRERAWALR	126	0.80
7	ISTVDARNLVPMVATV	104	0.78
8	FIAGNSAYEYVDYLFK	81	0.77
8	TEECQLPSLKIFIAGN	70	0.77
9	DNEILLGNHRTEECQL	60	0.73
10	NGSHTSRTTSAQTRSV	167	0.72
11	LFKRMIDLSSISTVDA	94	0.70
12	TIYNTTLKYGDVVGVN	200	0.69
13	AYEYVDYLFKRMIDLS	87	0.66
13	FFFDIDLSTSHATSST	150	0.66
13	YDPVAALFFFDIDLST	143	0.66
14	PMVATVQGQNLKYQEF	114	0.65
15	AVSHRANETIYNTTLK	192	0.64
16	NFANSSYVQYGQLGED	45	0.63

Table 9. HLA class I-restricted CTL epitopes of the chimeric proteins.

MHC Type	Epitope sequences	SYFPEITHI score
HLA-DRB1*1501 (DR2b)	A W A L R Q Y D P V A A L F F	34
HLA-A*26	E T I Y N T T L K Y	34
HLA-B*08	W P R E R A W A L	33
HLA-DRB1*0101	L R Q Y D P V A A L F F F D I	32
HLA-DRB1*0101	V P M V A T V Q G Q N L K Y Q	30
HLA-A*02:01	N L V P M V A T V	30
HLA-DRB1*1101	Y Q E F F W W P R E R A W A L	27
HLA-A*01	A T V Q G Q N L K Y	26
HLA-A*01	E T I Y N T T L K Y	26
HLA-DRB1*0101	F F W W P R E R A W A L R Q Y	26
HLA-A*02:01	T L K Y G D V V G V	25
HLA-A*01	P R E R A W A L R Q Y	25
HLA-A*11:01	A T V Q G Q N L K	25
HLA-A*26	E R A W A L R Q Y	25
HLA-A*68:01	E T I Y N T T L K	25
HLA-B*15:16	V S H R A N E T I	25
HLA-A*26	D P V A A L F F F	24
HLA-A*26	A T V Q G Q N L K Y	24
HLA-A*24:02	Q Y D P V A A L F	24
HLA-B*13	R Q Y D P V A A L	24
HLA-B*44:02	N E T I Y N T T L K Y	24
HLA-B*39:02	R Q Y D P V A A L	24
H2-Ld	D P V A A L F F F	24
H2-Ad	L R Q Y D P V A A L F F F D I	24
HLA-DRB1*0101	A R N L V P M V A T V Q G Q N	24
HLA-DRB1*0101	A W A L R Q Y D P V A A L F F	24
HLA-DRB1*0701	V Y S Q H V T S S E A V S H R	24
HLA-DRB1*1101	E F F W W P R E R A W A L R Q	24
HLA-DRB1*1501 (DR2b)	R N L V P M V A T V Q G Q N L	24
HLA-DRB1*1501 (DR2b)	S Q H V T S S E A V S H R A N	24
H2-Ad	S V Y S Q H V T S S E A V S H	24

Table 10. HLA class I-restricted CTL epitopes of the recombinant protein according to BIMAS server.

Sequence	Start Position	HLA type	Score
YLFKRMIDL	93	A_0201	836.253
ALFFFDIDL	148	A_0201	300.355
NLVPMVATV	111	A_0201	159.970
YLFKRMIDL	93	A_0205	126.000
RQYDPVAAL	141	A_0205	100.800
DYLFkRMIDL	92	A24	300.000
AYEYVDYLF	87	A24	210.000
QYDPVAALF	142	A24	168.000
QYDPvAALFF	142	A24	120.000
CYSRPVVIF	36	A24	100.000
KVLRDMNVK	22	A68.1	240.000
ETIYNTTLK	199	A68.1	180.000
CVTInQTSVK	13	A68.1	120.000
YLFKRMIDL	93	B14	100.00
TEECQLPSL	70	B60	160.000
NETIYNTTL	198	B60	160.000
RQYDpVAALF	141	B62	176.000
WPRERAWAL	132	B7	800.000
WPRERAWAL	132	B8	480.000
RQYDpVAALF	141	B_2702	300.000
SRPVVIFNF	38	B_2702	200.000
KRMIDLSSI	96	B_2702	180.000
RQYDPVAAL	141	B_2705	3000.000
LRQYdPVAAL	140	B_2705	2000.000
KRMIDLSSI	96	B_2705	1800.000
RQYDpVAALF	141	B_2705	1500.000
SRPVVIFNF	38	B_2705	1000.000
SRTTSAQTR	172	B_2705	1000.000

Theoretically, our chimeric construction consists of five putative epitopes and anigenic fragments that could be assembled together as a unique construct optimally appropriate for human expression system.‘‘GenScript’’ codon optimization technology can optimize any gene to deliver the highest protein expression level in any target host. During the translation, optimal codons are easily recognized by numerous tRNAs [56]. Multiple factors could affect the expression of chimeric genes in human systems, including immature polyadenylation, antiviral motifs, mRNA stability, aberration from the preferred codon-usage, and unusual splicing [20]. In order to enhance the mRNA stability, DNA motifs such as the potential polyadenylation signal sequence AATAAA and the ATTTA sequence that may havean influence on mRNA instability were omitted from the chimeric gene.

Studies have been reported that rare codon usage can create higher order of secondary structure, which might cause delayed ribosomal movement, so the high expression of chimeric protein can be reduced by aberration from proper codon usage [57]. Elimination of the rare codons is an approachthat is frequently used for solving this problem. For this, the recombinant gene was organized based on the codon usage of nuclear encoded genes of human. Codon adaptation index (CAI) measures the adaptiveness percentage of codon usage bias for a specific nucleotide sequence with a range of zero to one. Of course, It should be noted that nousual gene reaches ideal theoretical value (1.0) [58, 59]. Thus, in our synthetic gene the index was improved to 94 %. In addition, to increase the mRNA stability GC content was balanced to 58.98.The essential restriction enzyme recognition sites were inserted to 3' and 5' ends of chimeric gene for subsequent assays.

In order to specify the chimeric protein potential folding, the Mfold algorithm was used. This theoretical algorithm can find the best structure with theminimum energy and ΔG of the start codon. mRNA stability is an important factor in gene expression. Higher the stability leads to increased expression [60]. The lower ΔG of an mRNA means the more stability [44].

In 5' end of optimized mRNAs, the first ten nucleotides were examinedfor checking the suitabilityof the initiation of translation and ribosome binding. Since these ten nucleotides are not in secondary structure with $\Delta G < -10$, mRNA will be translated with high efficiency. So, the data from this predictive algorithm showed that the mRNA optimal structure was suitable for effective ribosome binding and translation initiation in the desired host.The Kozak consensus sequence (5'GCCACCATGGC) around the start codon on eukaryotic cell mRNA, can play an essential role in the precise initiation and efficiency of the translation process [52]. So in the chimeric construct before the start codon, the 5'GCCACC sequence was added.The evaluation of the protein secondary structure has an essential role in protein tertiary structure prediction with the ab initio and computational model or protein fold identification by offering additional restrictions [61]. One of the most popular schemes of secondary structure prediction, which provide high prediction accuracy, is the GOR method. This method considers not only the possibility of a particular secondary structure for each amino acid, but also the conditional probability of the amino acid supposing each structure given the contributions of its neighbors [36]. In this study, chiefly GOR method has been used. The physicochemical parameters of recombinant proteins were evaluated. The protein pI value (6.44) showed that the protein has an acidic nature. High extinction coefficient (33015) at 280 nm, defines the presence of high concentration of cystein. On the basis of instability index, Expasy's ProtParam classifies the chimeric protein as stable. The protein may be stable for a wide range of temperature because of high aliphatic index.The recognition of antibody–antigen interaction, conformational epitopes and tertiary structure is an essential principle for the normal design of vaccines and novel drugs [62]. In order to achieve this goal, ab initio I-TASSER online software was exploited [41]. In comparison to Swiss model, Zhang software predicted the three dimensional structure of protein more precisely [36].

TM-score and RMSD were used for the assessment of the predicted models. Expected TM-score of 0.52±0.15 confirms the correctness of the 3D model. A TM-score >0.5 shows an accurate topological model. The RMSD value was 9.0±4.6Å that means a good measure of accuracy. Other parameters including the C-score confirms high confidence for this model and the z-score refers to total model quality and measures the deflection of the structure energy according to an energy distribution obtained from random conformations.

Conclusion

According to our study, the results show that the protein structures were suitable, therefore, it can be expected that the construct is proper for practical experiments and is a good stimulator of humoral and cellular immune responses.

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