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ORIGINAL ARTICLE

Characterization of the conserved regions of E1A protein from human adenovirus for reinforcement of cytotoxic T lymphocytes responses to the all genogroups causes ocular manifestation through an in silico approach

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ABSTRACT

Background and Objectives: Adenovirus species B, C, D, and E are the most common causes of ocular manifestations caused by adenoviruses. FDA-approved treatment agents for adenovirus infections are not available. Cell-mediated immunity is the major protective mechanism versus human adenoviruses (HAdVs) infection and T cells specific for peptide epitopes from nonstructural proteins can prevent adenoviral dissemination. E1A CR2 region of HAdVs Epitopes predicted for reinforcing cytotoxic T lymphocytes (CTLs) in the EKC patients. Among human adenoviruses E1 protein, four distinct E1A regions had a significantly higher level of homology than the rest of E1A protein. E1A protein inhibits IFN signal transduction. Epitope-based vaccines are designed to have flexible and simple methods to synthesize a vaccine, using an adjuvant to trigger fast immune responses. CTL epitopes were applied to create a multiepitope vaccine. Conserve region1 (CR1) and CR3 have less antigenicity compared to CR2. Additionally, CR3 in HAdV-D8 contains three toxic areas. CR4 similar to the two regions CR1 and CR3 do not show acceptable antigenic properties.

Materials and Methods: Bioinformatics' tools were used to predict, refine and validate the 3D structure of the construct. Effective binding was predicted by protein-protein docking of the epitope vaccine with MHC-I molecules and revealed the safety and efficacy of the predicted vaccine construct.

Results: In silico analysis show that rising levels of cytotoxic CD8 + T cells, TH1 cells, macrophages, and neutrophils are linked to IFN-dominant TH1-type responses, which are detected in putative immune individuals.

Conclusion: Combined with 3D protein modeling, this study predicted the epitopes of E1A CR2 protein in HAdVs.

Keywords: In silico model; Adenovirus E1A proteins; Keratoconjunctivitis; Molecular docking; Cytotoxic T-lymphocyte

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INTRODUCTION

Human Adenoviruses (HAdVs) are common pathogens; induce various diseases in humans, such as pneumonia, genital tract infections, acute gastroenteritis and outbreak of epidemic keratoconjunctivitis (1-2).

In humans, currently, 104 unique HAdV genotypes (available at: http://hadvwg.gmu.edu) in seven species (Human adenovirus A to G) have been identified. Different types are associated with different conditions: respiratory disease (mainly species HAdV-B and C), conjunctivitis (HAdV-B and D), gastroenteritis (HAdV-F types 40, 41, HAdV-G type 52), obesity or adipogenesis (HAdV-A type 31, HAdV-C type 5, HAdV-D types 9, 36, 37) (3).

Even though the common point among all studies introduced human adenoviruses D species (types 8, 15, 37, 53, 54, 56, and 64) is usually caused of epidemic keratoconjunctivitis (EKC), nevertheless, several other HAdVs types, including species B HAdVs serotypes 3, 7 and 11, HAdVs-C 2 and C5, and HAdV-E4, have also been reported (4).

Although the majority of patients do not develop concomitant signs or visual sequela as a result of EKC, but a distinct subset of subepithelial infiltrates (SEIs) will demonstrate. Diffuse punctate epithelial keratitis, transient anterior uveitis and chronic complication such as anterior stromal infiltrates are the main complication, which may persist for months or even years. EKC have been associated with Epithelial Keratitis and vision loss as long term complications. In severe cases of adenoviral keratoconjunctivitis, corneal involvement in EKC occurs, varying in appearance as a keratitis, pseudomembrane formation, development SEI following the acute phase of the infection, decreased sensitivity to the cornea, subjective visual disturbances, corneal neovascularization formation, long-term sequelae and scarring (5, 6). HAdV-D8 has been linked to nosocomial outbreaks (7).

Recently, antiviral activity for systemic HAdVs infections has been extensively investigated and created the conditions for the more specific assessment of the eye manifestations of this virus. Depletion of T-cells can result in inadequate or insufficient cellular HAdVs immunity. The depletion and use of long-term immunosuppressant of T cells associated with stem-cell transplant and lack of congenital immunodeficiency T cells are a significant risk for persistent and severe infection with HAdVs (8).

The HAdVs genome is a 36 kb linear double-stranded DNA with 38 protein-coding genes (9). Several adenovirus genes modulate the host immune response by a variety of mechanisms. The Early-region1 (E1) products are, including E1A and E1B. E1A protein is the first viral protein in the infection of HAdVs and induces other early genes that facilitate viral replication, block IFN pathways and ISG expression induction, involved in transcriptional regulation, cell cycle progression, induction of apoptosis in infected cells, cellular proliferation and gene expression (10). Five mRNAs are produced by the E1A gene that encodes its proteins. The 13s and 12s mRNAs are two main products. The 13S-encoded protein contains four conserved regions (CR1-CR4) (11).

E1A performs many functions through linear protein-protein interaction motifs in the CRs and the large numbers of cellular proteins, such as IDMBR, CoRNR Box, pRB_AB Groove, TRAM, MYND, LxCxE, CKII, Acidic Stretch, CtBP, NLS (12).

Cell-mediated immunity is the most important protective mechanism against adenovirus infection. T lymphocytes specific for HAdVs epitopes are demonstrable in humans (13).

Non-structural proteins, in comparison to structural proteins, are not found in viral particles and cannot contribute directly to antibody-dependent stimulation. T-cells are an important element of the immune response necessary for viral infections' management, and these lymphocytes intend at epitopes highly conserved in non-structural proteins. Nonstructural epitopes are main targets for T lymphocytes, like what have been identified in the Zika virus (ZIKV) and Dengue virus (DENV) proliferation and intracellular replication regulation (14). Epitope-based vaccines are designed to have flexible and simple methods to synthesize a vaccine, using an adjuvant to trigger fast immune responses (15).

In summary, we present a designed epitope-based peptide vaccine in protection against E1A CR2 conserved region of HAdVs which may be a valuable alternative in order to control adenoviral dissemination infection.

MATERIALS AND METHODS

Ethics approval and sampling. The ethics committee approved this research of Ahvaz Jundisha-

pur University of Medical Sciences (IR.AJUMS. REC.1397.362) with the ethical principles stated in the Declaration of Helsinki. Five HAdV-D8 positive EKC samples were selected to evaluate in silico prediction of T-cell epitopes. Adenovirus DNA was extracted then the full length E1 gene were amplified and sequenced.

Retrieval of protein E1A sequences. The E1A protein sequence of human adenovirus B (types 3,7 and 11), adenovirus C (type 2 and 5), adenovirus D species (types 8,15, 37, 53, 54, 56, and 64) and adenovirus E (type 4) was collected from the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/) and processed using various computational and bioinformatics tools and algorithms. Determining the conserved regions (CRs) was performed using the multiple sequence alignment (MSA) program. Different characteristics, such as physiological, structural, functional, and epitope prediction, were evaluated using online servers or databases listed in Table 1. The following was briefly mentioned in each procedure.

Antigenicity prediction in the E1A protein. The highest levels of antigenic protein CRs were defined on the VaxiJen server. CRs with a VaxiJen score ≥ 0.4 have been selected as the candidate epitopes regions.

Prediction of CD8+ cytotoxic T lymphocytes (CTLs). Different online servers have been explored to predict CTL epitopes. CTL epitope mapping was chosen by NetMHCpan-4.1 and IEDB online tools (NetMHCpan EL 4.1 method). The CTL epitopes (binding allele's sequence of MHC I) were checked using ProPred 1 server. The predicted epitopes were checked for their antigenicity, allergenicity, and toxicity by VaxiJen AllerTOP v. 2.0 and ToxinPred servers, respectively.

Epitope conservation analysis. Epitope Conservancy Analysis of the IEDB server was applied for computing and predict the degree of the conservancy of the best score epitopes within the CR2 region of the E1A protein sequence set. Sequences with the highest identity were exerted for developing multi-epitope

Table 1. List of online Servers or Databases were used for design of epitope vaccine

N	Servers	URLs and Links	DOI of references
1	MSA program	https://www.ebi.ac.uk/Tools/msa/clustalo/	10.1093/nar/gkt376
2	VaxiJen	http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html	10.1186/1471-2105-8-4
3	ESPript 3.0	http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi	https://doi.org/10.1093/nar/gku316
4	IEDB Epitope Prediction	http://tools.iedb.org/mhci/	10.1093/nar/gkz452
5	NetMHCpan-4.1	http://www.cbs.dtu.dk/services/NetMHCpan-4.1/	10.1093/nar/gkaa379
6	ProPred 1	http://crdd.osdd.net/raghava/propred1/	10.1093/bioinformatics/btg108
7	AllerTOP v. 2.0	https://www.ddg-pharmfac.net/AllerTOP	10.1007/s00894-014-2278-5
8	ToxinPred	http://crdd.osdd.net/raghava/toxinpred	10.1371/journal.pone.0073957
9	IEDB Analysis	http://tools.iedb.org/conservancy/	10.1186/1471-2105-8-361
10	pHLA3D	https://www.phla3d.com.br	10.1016/j.humimm.2019.06.009
11	RCSB PDB	https://www.rcsb.org/	doi.org/10.1093/nar/28.1.235
12	AlgPred	https://webs.iiitd.edu.in/raghava/algpred/submission.html	10.1093/nar/gkl343
13	SOPMA	https://prabi.ibcp.fr/htm/site/web/services/secondaryStructurePrediction	10.1016/0022-2836(78)90297-8
14	ProtParam	https://web.expasy.org/protparam	10.1385/1-59259-890-0:571
15	SolPro	http://scratch.proteomics.ics.uci.edu	10.1093/bioinformatics/btp386
16	I-TASSER	https://zhanglab.ccmb.med.umich.edu/I-TASSER/	10.1038/nprot.2010.5
17	3D Refine	http://sysbio.rnet.missouri.edu/3Drefine/	10.1093/nar/gkw336
18	Molprobity	http://molprobity.biochem.duke.edu/	10.1002/pro.3330
19	ERRAT	https://servicesn.mbi.ucla.edu/ERRAT/	10.1002/pro.5560020916
20	ClusPro	https://cluspro.bu.edu/publications.php	10.1093/bioinformatics/btx216
21	PyMOL	https://pymol.org/	10.1002/wcms.1298
22	C-IMMSIM	https://kraken.iac.rm.cnr.it/C-IMMSIM	10.1371/journal.pone.0009862, 2010
23	JCat	http://www.jcat.de/	10.1093/nar/gki376
24	Snapgene	https://www.snapgene.com/	-

vaccines.

Retrieved HLA-I molecules from the protein data bank. The HLA I molecule were retrieved from the Protein Data Bank, RCSB PDB database, and afterward, the pHLA3D, which online database of HLA molecules predicted 3D structure was used to verify the selected structures.

Designing the construct of the multi-epitope based vaccine. For constructing a multi-epitope vaccine construct, the selected best Cytotoxic T cell epitopes were joined by a Flexible GPGPG linker. For the better immunogenic response, a cholera toxin B subunit (CTB) adjuvant was added by a rigid EAAAK linker into both sides of the vaccine construct. In order to the isolation and purification of the vaccine construct, 6xHis tag was added by GPGPG linker at the C-terminal.

Assessment of antigenicity, allergenicity and toxicity of the vaccine construct. For the antigenicity evaluation of the vaccine construct, VaxiJen tools have been utilized. Two servers, AlgPred and AllerTop, were exploited for the allergenicity assessment of the multi-epitope vaccine construct. Additionally, to verify non-toxicity; the ToxinPred database was applied.

Prediction of secondary and tertiary structures of the vaccine construct. 2D structure was predicted by a new method known as SOPMA (self-optimized prediction method) that has recently been described to improve the accuracy rate for predicting the secondary protein structure. Analysis of the physicochemical properties of the multi-epitope vaccine construct was utilized by ProtParam. Protein solubility was estimated using the SolPro tool available on the SCRATCH Protein Predictor Site. Iterative Threading ASSEmbly Refinement (I-TASSER) server is an integrated platform and 3D structure prediction for automated protein structure, which was explored to approach the functional prediction of 3D multi-epitope vaccine structure.

The predicted structure was refined utilizing the protein structure refinement server, 3D Refine, and finally; the construct was assessed by Ramachandran diagram analysis of the model by Molprobity web server. Moreover, the Quality Factor and verification of 3D construct the structure was identified by the ERRAT data center.

Vaccine construct HLA docking analysis. Analysis of Interaction between the predicted vaccine construct and HLA-1 molecule was carried out by the protein-protein docking server ClusPro. ClusPro Docking scores, which displayed energetically favorable, was compared with a neutral molecule such as albumin. Stable interaction of the vaccine constructs with HLA-1 molecules, which were considered recognized by a wide range of HLA alleles, could be eligible for therapeutic vaccines. Visualization of the results was accomplished using the PyMOL molecular visualization system.

Immune simulation. The modeling of immune simulation was reliable using the CmmSim-I immune simulation server with a simulated level of IFN-γ, Natural Killer cells (NK cells), macrophages, epithelial cells, CTL, and concentrations of other cytokines and interleukins after three-time immunization given four weeks apart.

Codon optimization and in silico cloning. Codon optimization was done using the JCat server to express the ultimate of the epitope vaccine candidate in the *Escherichia coli* K12 strain (*E. coli* K12). Extra alternatives were selected to prevent enzyme restriction cleavage sites; rho-independent transcription termination and prokaryote ribosome-binding site. The JCat performance includes the codon adaptation index (CAI) and GC content ratio, which can be used to determine levels of protein expression.

E. coli K12 strain was selected as a host to clone the optimized gene sequence of the final multi-epitope vaccine. The expression vector pET28a (+) was cleaved by BamHI and HindIII restriction enzyme sites, which had been added respectively to the N and C sequence terminals. The 6X histidine tag was inserted at the end of 3 ' gene for isolation and purification and subsequently, was used the SnapGene software to ensure vaccine construct could be an expression.

RESULTS

Retrieval of adenovirus non-structural E1A protein and antigenicity prediction. The complete E1A protein sequence ofHAdV-D8 with accession number

QBZ81857 which deposited in GenBank along with other HAdVs protein sequences, retrieved from the NCBI protein database. The conserved regions of 13s E1A protein sequence among all genotypes in Human adenoviruses were assessed and determined by the Multiple Sequence Alignment (MSA) software (Fig. 1). Conserve sequences were explored by the VaxiJen server for their antigenic nature. The antigenic CRs with the antigenicity score ≥0.4 were taken for epitope screening. Overall prediction for the protective antigen of complete adenovirus 13s E1A protein sequence, 0.5345, and was detected as probable antigen. Each CRs have analyzed through the VaxiJen server and relieved that CR2 with 0.8010 antigenicity scores was the best-conserved region on all genotypes in the E1A protein of human adenoviruses. CR2 region residue with acceptable antigenicity scores have the highest conservative (Fig. 2) and antigenicity among all genotypes in E1A protein of human adenoviruses. Table 2 identifies the antigenicity score of four CRs in the E1A region and was recognized after the MSA for all seven species. Overall, prediction for the protective antigen score in the CR1 and CR3 was weak and equivalent 0.5409 and 0.4667 respectively. Furthermore, about CR4, was no observed acceptable antigenic nature. GCKSCQYHRE, DPNASCALCY, ESSPLEEDHP were recognized as three toxic domains in HAdV-D CR3.

Epitope screening and conservancy analysis. Antigenicity, allergenicity, and toxicity were evaluated for the predicted epitopes by online tools (Table 1), and the appropriate epitopes were considered. The maximum immunogenicity of all epitopes has been identified to ensure optimum binding affinity. The E1A-CR2 sequence was also used to predict MHC class I binding regions utilizing the online servers (Table 3). For MHC binding affinity and to cover the most of the immune responses to CTL epitopes with MHCI, percentile rank the IEDB team recommended <= 1% for each combination. CTLs play a central role in the removal of pathogen-infected cells and tumor growth. CTLs screen biological membranes for MHC type I-peptide complex derived from viral proteins and eradicated infected cells. NetMHCpan-4.1, IEDB, and ProPred1 online tools forecast the epitopes for MHC Class-I, and the sequences indicated in Table 3 were assigned for this purpose. This analysis of the epitopes in the IEDB epitope conservation analysis resource showed the conserved and variable residues of the epitopes in the E1A-CR2 sequences of other countries. Between 98 sequences retrieved from the NCBI database, epitopes selected above show the highest conservancy according to the Fig. 2 (the 91.67 percent to 100 percent range).

Demographic and coverage analysis of HLA alleles. Design of vaccines in different population depends on the relationship between the epitopes and the percentage of HLA alleles. Across the world, the expression of MHC molecules differs based on the race. Also the distribution of MHC molecules varies by ethnicity around the world. In order to check the population coverage of the vaccine model, the presence of specified MHC alleles was evaluated. The 3D structure of HLA-A*0201 with PDB ID 4UQ3 (by method X-Ray diffraction 2.1 Å) was retrieved from PDB database. Subsequently, verification and validation were performed in the pHLA3D online server.

Designing of multi-epitope based vaccine. The selected best CTL epitopes were applied to create a multi-epitope vaccine using a GPGPG linker. CTB adjuvant was applied to the multi-epitope based vaccine model using an EAAAK linker to enhance immunogenic response.

Prediction of secondary and the tertiary structures of the vaccine construct. The secondary structure of the multi-epitope vaccine made up of 368 amino acids (aa) which were predicted by GOR4 method of SOPMA server (Fig. 3) are composed of 122/368 aa (33.15%) alpha helix, 66/368 aa (17.93%) extended strand, 180/368 (48.91%) random coil. The vaccine construct's molecular weight as predicted by ProtParam server was 40 Kd. The average half-life was observed in mammalian reticulocytes 30 hours, in yeast> 20 hours and in *Escherichia coli*> 10 hours. The Aliphatic Index and the Hydropathic Grand Average (GRAVY) were 77.74 and -0.379 respectively. Predicted solubility upon overexpression was obtained by SolPro was 0.997342. The 3D structures of the model were obtained by I-TASSER with C-score -1.34, Exp.TM-Score 0.55+-0.15, Exp.RMSD 9.7+-4.6 and cluster density 0.1099 submitted for the refinement process by 3D refine (Protein Structure Refinement Server). The qualities of the 3D structures were improved after refinement according to the results of the Ramachandran plot by Molprobity web server. The Ramachandran plot was shows 89.9% of

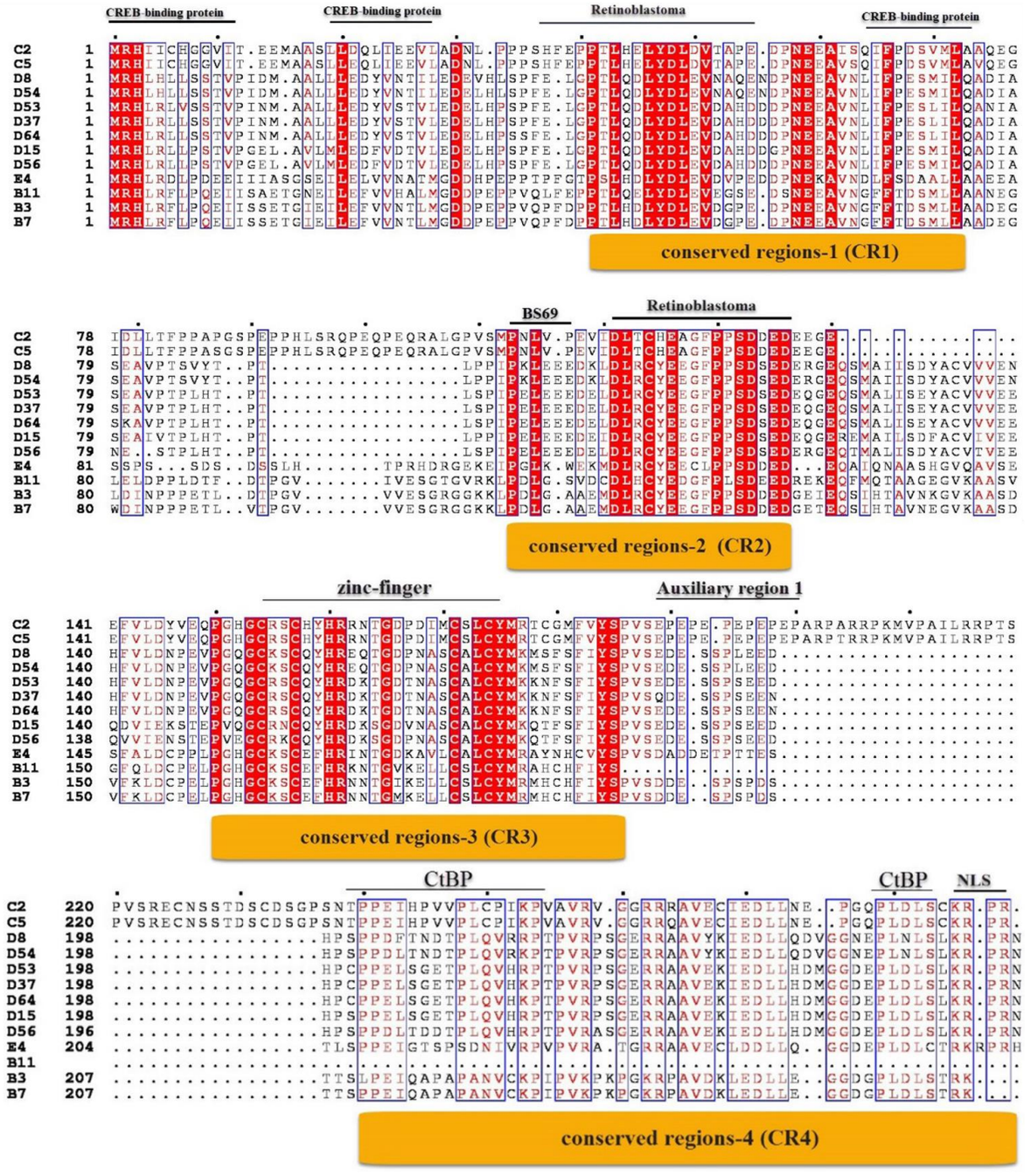

Fig. 1. E1A protein adenovirus sequences alignment. By ESPript3.0 online tools,gaps are indicated as dots. Red and reddish colors indicate higher levels of conservation (>70% similarity). The region interacts with Rb, zinc-finger, CtBP, CBP, AR1, NLS and BS69 are indicated. Yellow Arrowheads indicate highly conserved residues. The positions of the conserved regions (CR) are indicated as yellow bars. Sequence conservation regions are indicated as CR1, CR2, CR3, and CR4 between D, B, C and E Genotypes of adenoviruses. Numbers at the first of each row indicate the last residue's position within the particular sequence.

Fig. 2. A graphical representation of E1A-CR2 multiple sequence alignment powered by web based application, WebLogo among all genotypes in Human Adenovirus

Table 2. List of E1A proteins CRs used for antigenicity prediction by VaxiJen serve

Conserve Regions	Genotype of Human Adenoviruses	Antigenicity Score*	Antigenicity Prediction using VaxiJen server
CR1	C5, D8, B7, E4	0.3282, 0.5584, 0.0013, 0.1280	-/+/-/-
CR2	C2, C5, D8, D15, D37, D53, D54, D56, D64, B3, B7, B11, E4	0.9820, 0.9820, 1.2208, 1.1890, 1.1833, 1.1833, 1.2208, 1.1833, 1.1833, 1.2308, 1.2308, 0.9488, 1.2841	Probable antigen +/+/+/+/+/+/+/+/+/+/+/+/+
CR3	C5, D8, B7, E4	0.5787, 0.7145,0.3412,0.3631	+/+/-/-
CR4	C5, D8, B7, E4	0.5981,0.1819,0.2773,0.3864	+/-/-/-

*Antigenicity scores were analyzed using VaxiJen at threshold 0.4 to check the antigenicity

Table 3. MHC class I binding peptides on the basis of antigenicity, allergenicity and toxicity

HAdVs Genotype[1]	Candidate Epitopes	VaxiJen Score[2]	Allele	Allergenicity and Toxicity
D8,54	KLDLRCYEE	2.2433	HLAB*44:02, HLAB*44:03, HLAA*01:01, HLAB*15:01, HLAA*30:02, HLA-B*40:01, HLA-A*26:01, HLA-B*53:01, HLA-B*35:01, HLA-A*68:01, HLA-A*30:01, HLA-A*33:01, HLA-A*11:01, HLA-A*03:01, HLA-B*51:01, HLA-B*58:01, HLA-B*57:01, HLA-B*08:01, HLA-A*68:02, HLA-A*31:01, HLA-A*32:01, HLA-A*02:01, HLA-A*02:03, HLA-B*07:02, HLA-A*02:06, HLA-A*23:01, HLA-A*24:02	Non-allergen Non-Toxin
D37,53,56,64	ELEEEDELDL	1.2276		
D15	ELEEEDEIDL	1.2328		
C2, C5	LVPEVIDLTC	1.6485		
B3,7	DLGAAEMDL	1.5401		
B11	YEDGFPLSDE	0.4623		

[1]There was no acceptable epitopes in the CR2 region of HAdV-E4

[2]Overall Prediction for the Protective Antigen for selected epitopes using VaxiJen server (Threshold =0.4)

all residues were in allowed regions, while the overall quality factor of ERRAT is 79.8% and 92.93% of the residues have averaged 3D-1D score >= 0.2.

Relevance of the HLA-epitope and docking. Protein-protein docking was performed to verify the interaction of the designed vaccine with the cleft of MHC classes there by Protein - protein docking of the constructed vaccine and MHC classes was carried out by ClusPro server. ClusPro Docking scores were demonstrated the effective binding of the multi-epitope construct vaccine and MHC classes. Table 4 exhibited complex of Docking, Members of residues participate in the reactions, Representative of energy and Weighted Score.

Immune simulation. Cytokine levels, increased numbers of cytotoxic CD8 + T cells, Natural Killer cells, T-regulatory lymphocytes after injections are seen in the plot of Fig 4. Interferon gamma (IFN-), a cytokine involved in both innate and adaptive immune responses, stimulates and increases natural killer cells

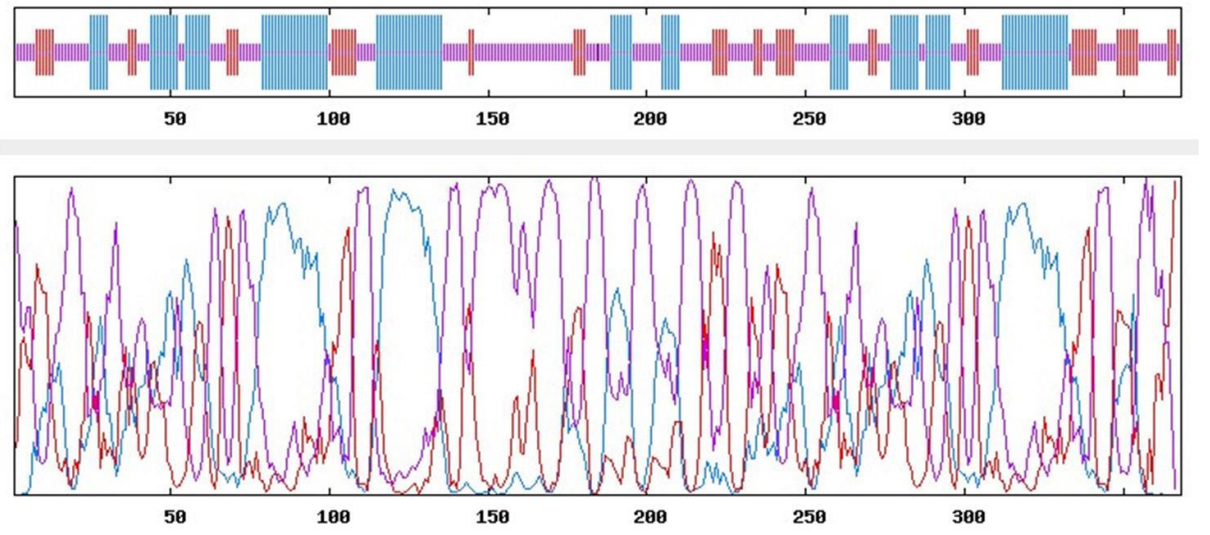

Fig. 3. GOR4 secondary structure prediction:Secondary structure analysis of Vaccine Construct using SOPMA and GOR4 prediction software. Alpha helix (blue), Extended strand (red) and Random coil (purple)

Table 4. Model scores of ClusPro Docking

Complex of Docking	Members*	Representative	Weighted Score
Albumin -MHC I	41	Lowest Energy	-576.8
		Center	-576.8
Construct Vaccine- MHC I	67	Lowest Energy	-1041.3
		Center	-930.2

*Members of residues participate in the reaction of docking complex of proteins (multi-Epitope Vaccine Construct or albumin) with MHC I.The cluster "center" is the 'average' pose representative of the cluster, on the other hand the structure that has the highest number of neighbor structures in the cluster and the "lowest energy" is the pose within this cluster that has the lowest energy.

and macrophage responses to antigen presented by MHC molecules. The insert plot in the Fig. 4 shows the amount of IL-2 with the Simpson index, with the blue color.

Indicating D. D index is a diversity metric. D rises over time reflect the development of different T-cell epitope-specific dominant clones. The lower the D value reflects the smaller the diversity. The anergic stage is the tolerance of the T-cells to the antigen leading to frequent stimuli while the resting phase reflects cells that do not present an antigen. Refer to E part of Fig 4.

Codon optimization and in silico cloning. Codon optimization was performed using JCAT server which designed for improved protein expression. The CAI (codon adaptation index) value of CDNA was found to be 0.603 before adaptation, and GC content was 54.2%. After adaptation, the CAI score was increased to 0.96 with 52.6% GC content. Ultimately, in silico cloning of the E1A adenovirus epitope vaccine construct cDNA, which optimized for enhanced protein expression in *E. coli* K12 strain was inserted into the pET28a (+) vector through the restriction sites HindIII and BamHI (Fig. 5).

DISCUSSION

The purpose of the study was to predict the T cell epitopes of the E1A protein CR2 region in human adenovirus which causes ocular manifestation.

The EKC syndrome is distinct from the common mild adenoviral conjunctivitis. Pain, lacrimation, and photophobia are some of the more extreme symptoms of EKC (16, 17). The involvement is usu-

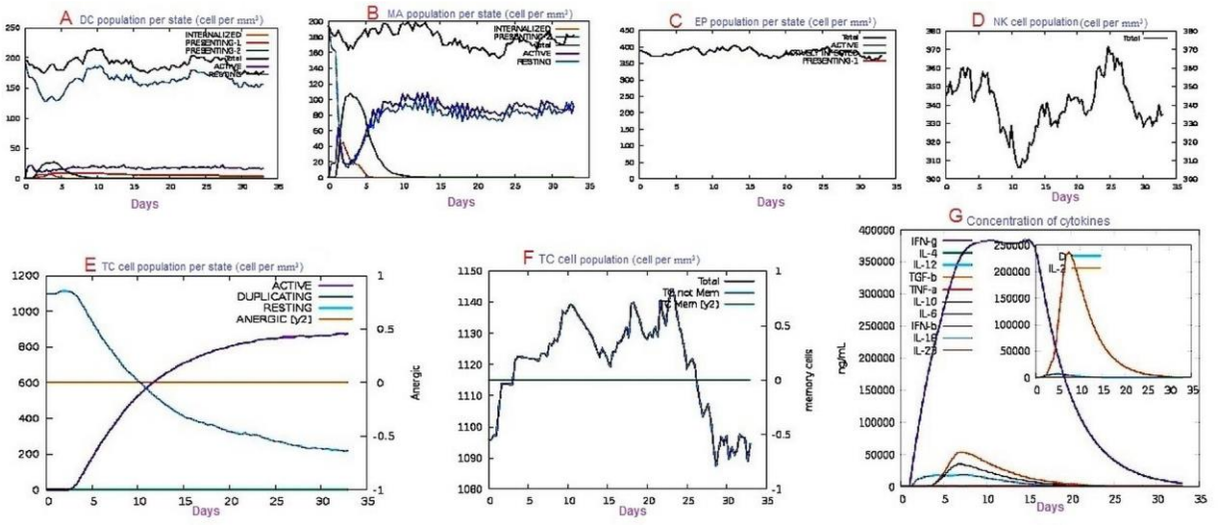

Fig. 4. C-ImmSim Introduction of a Vaccine Construct by in Silico Immune Simulation.
(A) Dendritic cells. DC can present antigenic peptides on MHC molecules. The curves show the total number broken down to active, resting, internalized and presenting the ag.
(B) Macrophages. Total count, internalized, presenting, active and resting macrophages.
(C) Epithelial cells. Total count broken down to active, virus-infected and presenting on Class-I MHC molecule.
(D) Natural Killer cells (total count).
(E) CD8 T-cytotoxic lymphocytes count per entity-state.
(F) CD8 T-cytotoxic lymphocytes count. Total and memory shown
(G) Concentration of cytokines and interleukins. D in the inset plot is danger signal. The increase in interferon-gamma after vaccination is shown in increments by green color in the plot

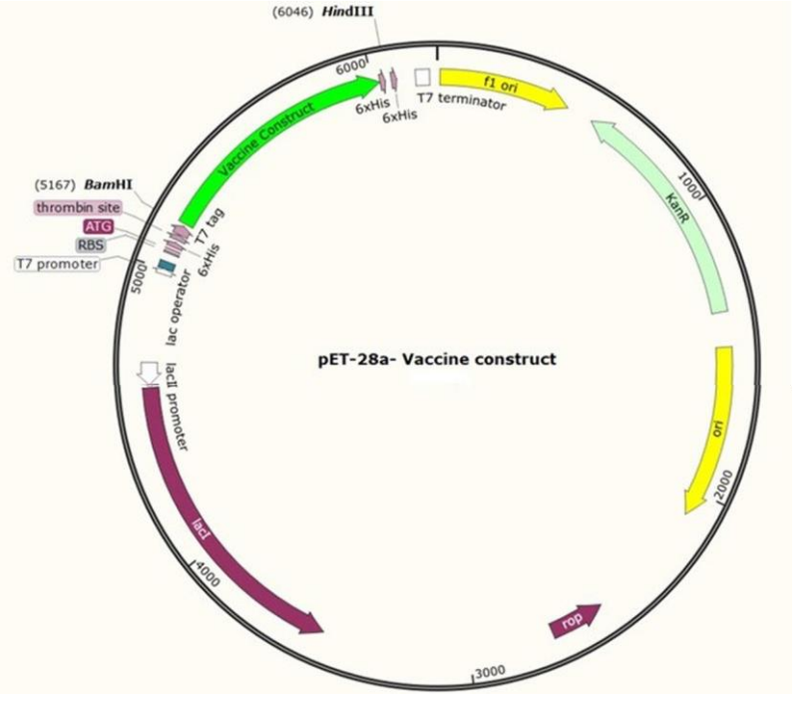

Fig. 5. Vaccine construct. Snap gene, was used for the document and visualize vaccine constructs. The expression vector pET28a (+) was cleaved by BamHI and HindIII restriction enzyme sites which had been added respectively to the N and C sequence terminals

ally unilateral, but it can spread to both eyes. Inflammation could be serious, resulting in sub corneal opacities, scarring, and vision loss. EKC is highly contagious and has been associated to the serotypes Ad8, Ad19, Ad37, and, less frequently, others (18). By inhibiting cytoplasmic signaling pathways and activating IFN-stimulated genes, human adenovirus blocks the IFN response (19).

In silico analysis in our study demonstrated that, rising levels of IFN-dominant TH1-type responses are associated with cytotoxic CD8+ T cells, TH1 cells, macrophages, and neutrophils which are stimulated in three doses. The study by Cecilia et al. showed that people infected with adenovirus D are more likely to have SEI development and long-term infection compared to other types of adenoviruses. They documented widespread geographic dissemination of viral types involved in the EKC infection. While 90% of EKC cases in Sri Lanka were due to adenovirus D species, with almost 80% of these specifically caused by adenovirus D-8. In the U.S., 45% of cases were adenoviral negative, with only 52% of cases resulting from adenovirus D infection, and of these, only 10% were caused by adenovirus D-8 infection. This reflects a shifting landscape for viral causes of keratoconjunctivitis at various geographical locations (20).

Reactivation of persistent latent human adenovirus is likely made possible by blocking the two types of interferon (IFN) response, known as type I and type II, that are required to help block expression of the HAdVs E1A gene. Restricting the IFN response enables the expression of the HAdVs E1A gene that leads to the replication and reactivation of HAdVs DNA in permissive cells and spread of HAdVs infection subsequently (19).

Cell-mediated immunity is the major protective mechanism versus adenovirus infection. Infusion of in vitro expanded of the CTL lines has led to successful therapy for refractory disease and decrease of adenoviral loads. Adoptive T-cell treatment is one of the most promising treatments for HAdVs. The process of adoptive T cell therapy includes leukocyte harvesting that is activated and extended in vitro using peptide-MHC I tetramers, rather than using lymphocytes extracted from the same donor as the transplant. The above treatment, with an antiviral use, appears to be complementary and effective (5).

A number of research groups have demonstrated that adoptive transfer of T-cells is an effective treatment for adenovirus infection. In an in vitro experiment has been reported to illustrate the capacity of anti-viral activity of HCM¬V-specific T cells (21). Another an in vitro study by Anna keib et al. shows that T cells specific for epitopes from both nonstructural and structural proteins can inhibit adenoviral dissemination. It was discovered that peptide-specific T cells in the nonstructural protein E1A prevents the spread of adenovirus to autologous monocytes. This prevention of viral spread demonstrates both the protective capacity of specific T cells and antiviral activity. The data obtained by these researchers indicate that 32 percent of T-cells specific to the LLDQLIEEV epitope amino acid sequence of the E1A inhibited adenoviral propagation. Their results showed that even low levels of specific T cells can accelerate to control the infection and recommend that the performance of the T-cell response is critical to prevention (22).

Despite the availability of effective treatments, the limitations of labor, time, and cost were essential to generate individual CTL lines for each patient limit the availability of such therapies to highly specialized centers. Thus, the use of vaccine technology with epitope base stimulating response of CTL can be considered as one of the best therapeutic choices (23).

In addition to structural proteins, use of nonstructural proteins can help improve the production of an effective vaccine in the long term by neutralizing the virus mutation rate (24, 25).

According to the Table 2, both domains CR1 and CR3 have less antigenicity compared to CR2. Additionally, CR3 in HAdV-D8 contains three toxic areas. CR4 similar to the two regions CR1 and CR3 do not show acceptable antigenic properties in our bioinformatics studies. CR2 of C5 (residues 93–139), D8 (residues 83–138), HAdV-B3 (residues 85–150), and E4 (residues 91–145) (26).

T cells play a crucial role in triggering both adaptive and innate immune activity to foreign particles. They are also the only factors for developing immune memory, which generate an effective and long-lasting immune response. Thus, the vaccinology strategy to design a novel epitope vaccine was focused on the proteome screening for the epitopes with high-affinity, which can trigger CTL responses. The binding stages of the MHC-epitope are vital and have a high sensitivity and specificity. Therefore, they are regarded to be one of the primary priorities

for vaccine development. MHC classes show a wide diversity due to the existence of many genes in the genetic code of humans; however, their frequency globally is not identical (27).

MHC alleles is a highly insightful framework, and various investigations have been carried out on the expression and distribution of HLA alleles in various individuals. The results of some studies showed that the dissemination of HLA-A types among the Iranian population was equivalent to that of the Caucasians. The most common alleles found in Caucasians were A*02 (27%) and -A*24 (10.8%), in six different Chinese population's HLA-A*02 (15.45- 30.65%), -A*11 (16.66-30.72%), and -A*24 (11.03-17.07%), in Korean Population A*02 (29%) and A*24 (22.4%), in a Berber population from North Morocco HLA-A*0201 and -A*0101, and in Armenian population, HLA-A*0201 (15.5%), -A*0101 (12.5%), and -A*2402 (12%) (28).

Vaccines with multiple epitopes are often poorly immunogenic and require coupling of chemical mixture of antigens and small molecular adjuvants (29). CTB's reduce the minimum concentration of antigens required for activation of immune cells and strong affinity to the GM1 (monosialotetrahexosylganglioside) receptor, which is predominantly found in epithelial mucosal cells (30). In a previous study, CTB was used as a multi-epitope vaccine against *Helicobacter pylori* and anti-atherosclerosis (31).

Flexible GPGPG linker dynamically separates CTL to preserve their appropriate 3D structures and enables both to move independently of each other. On the other side, a rigid EAAAK linker maintains the fusion epitopes and CTB adjutant were isolated and results in a better presentation of the structure.

A BLAST search of the selected epitopes against the NCBI database revealed a high degree of conservation among the E1A protein of adenoviruses. MSA of the predicted CTL epitopes also demonstrated a high level of conservation throughout identified homologous E1A proteins. 92.93% of the residues have averaged 3D-1D score >= 0.2. Given that should at least 80% of the amino acids have scored >= 0.2 in the 3D/1D profile for passing the verified 3D. So, the three-dimensional structure is acceptable.

In silico immune simulations were performed using the C-ImmSim server to further evaluate the immune response profile and immunogenicity of the epitope vaccine candidate. Increased numbers of cytotoxic CD8 + T cells, Th1 cells, macrophages and neutrophils are correlated with interferon-γ (IFNγ)-dominant Th1-type responses, identified in putative immune individuals (32).

Instead of predicting the conformations with the lowest energy, ClusPro selects the centers of highly populated clusters of low energy structures (33).

Annemieketh et al. observations were helpful in developing immune intervention schemes for autoimmunity and cancer. To induce a lasting antitumor CTL response, peptides Ad5E1A 234–243 (sequence SGPSNTPPEI; E1A peptide)/CD40 vaccination is used. In their experiments, vaccination with peptides in PBS would not induce CTLs specific for E1A unless combined with CD40 activation *in vivo*. The CTL response was long-lasting and strong (34).

In our study, significantly elevated levels of Natural Killer cells, T-cytotoxic cells, T-helper, IL-2 and INF-γ were detected by immune simulation. A further important finding was that IFN-γ and IL-2 levels increased after the first injection and continued at the highest level after repeated antigen exposure. A molecular visualization system, PyMOL, was used to present the results (Fig. 6).

Disinfection with povidone-iodine, or administration of ganciclovir, to reduce the viral load is not an adequate treatment. Since the severe or prolonged infections can cause permanent scarring to the cornea, resulting in vision loss and blindness (35). Thus, to prevent blindness, it seems necessary to design an EKC vaccine.

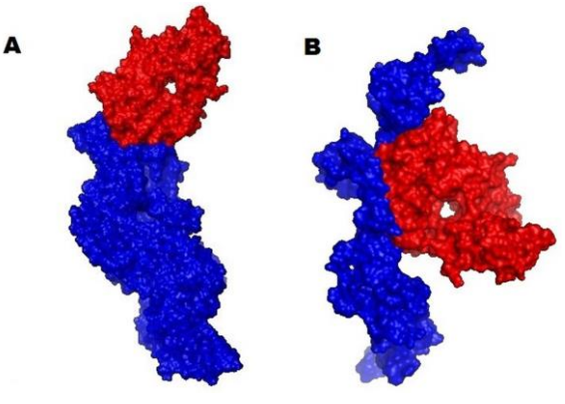

Fig. 6. Docking results: (A) Docking of Albumin (PDB id: 4f5s) with MHC I molecule (PDB id: 4UQ3) and(B) Multi-Epitope Vaccine Construct docking with MHC I molecule (PDB id: 4UQ3)

CONCLUSION

This study was accompanied by the prediction of the epitopes of the E1A CR2 protein of HAdVs together with 3D protein modeling. The research exposed possible T-cell epitopes that might inform the wanted immune response to the protein E1A CR2. These epitopes are very vital. They are useful as well as can likewise, aid in the growth of efficient vaccines.

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