In Silico Analysis of Synaptonemal Complex Protein 1 (SYCP1) and Acrosin Binding Protein (ACRBP) Antigens to Design Novel Multiepitope Peptide Cancer Vaccine Against Breast Cancer

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Abstract

Multiepitope cancer vaccines are manifesting as the future of breast cancer immunotherapy. In the present study, high immunogenic fragments of synaptonemal complex protein 1 (SYCP1) and acrosin binding protein (ACRBP) antigens were selected according to MHCs binding affinity and CD8⁺ cytotoxic T lymphocytes' (CTL) epitopes by various immunoinformatic servers. (RCQHKIAEMVALMEKHQYDKI) residue from p702–724 SYCP1 and the (YIQYPNYCSFKSQQL) residue from p481–496 ACRBP were selected as the immunodominant fragments. Then, the selected fragments were fused together with a furin-sensitive linker to form the final peptide vaccine construct. The cleavage sites, TAP transport efficiency, CTL epitopes, post-translational modifications, and B cells epitopes were predicted for the final construct. The final construct contained appropriate CTLs epitopes and also, several linear and conformational B cell epitopes. Also, it exhibited 90.37% HLA population coverage in the world. Therefore, these preliminary results require validation by in vitro and in vivo experiments.

Keywords Immunoinformatic · Cancer/testis antigen · SYCP1 · ACRBP · Multiepitope peptide vaccine · Breast cancer

Abbreviations

SYCP1	Synaptonemal complex protein 1
ACRBP	Acrosin binding protein
TAA	Tumor-associated antigens
CTA	Cancer/testis antigens
MHC	Major histocompatibility complex
NCBI	National Center for Biotechnology Information
CAI	Codon Adaptation Index
CTL	Cytotoxic T cell lymphocyte
HLA	Human leukocytes antigen

TAP	Transporter associated antigen presentation
PTM	Post-translational modifications

Introduction

Breast cancer is the leading cause of cancer death among women (Khanfir et al. 2013). Although breast cancer treatments have been considerably improved in recent years, patients' prognosis, especially for metastatic breast cancer, is not satisfying (Arpino et al. 2015; Redig and McAllister 2013). Tumors arise from a combination of genetic and epigenetic changes which lead to new cellular characterizations like immortality and uncontrolled proliferation (Erenpreisa and Cragg 2013; Ghahremani et al. 2018). These transformations cause new antigens expression in malignant cells. In addition, the immune system has the ability to recognize these changes and eliminate the cancer cells (Schumacher and Schreiber 2015). However, some cancer cells get the chance to escape the immune system's recognition (Whiteside et al. 2011; Beatty and Gladney 2015). Therefore, appropriate antigen selection and presentation to the immune system can effectively stimulate the immune response (Shastri et al. 2002).

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Vaccination is an effective strategy for introducing specific tumor antigens to the immune system. Many different types of vaccines including peptide and DNA vaccines have been utilized for breast cancer treatment (Vinay et al. 2015). DNA vaccines have exhibited appropriate stability, simplicity, and ability to introduce several antigens and epitopes to the immune system (Yang et al. 2014; Tiptiri-Kourpeti et al. 2016). Clinical trials of DNA vaccines against HER2 (Norell et al. 2010) and mammaglobin (Tiriveedhi et al. 2013) have shown promise in stimulating immune responses against breast tumor antigens (Yang et al. 2014). Also, peptide vaccines have gained lots of attention for breast cancer treatment. Vaccines containing derivative epitopes of breast tumor antigens such as mucin 1, human epidermal growth factor receptor 2 (HER2/neu), and carcinoembryonic antigen (CEA) are under investigation for use against breast cancer. They have been shown to provide immunogenic value without causing autoimmunity in phase I/II/III clinical trials (de Peres 2015; Thundimadathil 2012; Finn 2003). Although the peptide vaccine therapy shows encouraging responses, there are still challenges with respect to the variety of evasion mechanisms of tumor cells from the immune system (de Peres 2015; Karkada 2013). Therefore, there is a need to improve the immunogenicity of peptide cancer vaccines.

Appropriate selections of antigens for cancer immunization can profoundly affect the cancer vaccine efficacy. Tumor-associated antigens (TAAs) are the main utilized antigens for designing cancer vaccines (Vigneron 2015). TAAs are consisted of a vast variety of antigens including differentiation antigens (e.g. Melan-A/MART-1) (Coulie et al. 1994), mutational antigens (e.g. CTNNB1) (Robbins et al. 1996), overexpressed antigens (e.g. HER2) (Kraus et al. 1987), viral antigens (e.g. human papillomavirus proteins) (Demetris Iacovides et al. 2013), and cancer/testis antigens (CTAs) (Lurquin et al. 1997; Chomez et al. 2001). CTAs (e.g. SYCP1, ACRBP, NY-ESO-1, SSX2, etc.) are one of the most well-known TAAs. They are named after their restricted expression in testicular and cancerous tissues (Tarnowski 2016). Therefore, CTAs can be appropriate choices for anti-cancer immunization according to many cancer vaccines clinical trials (Zajac et al. 2017; Gjerstorff et al. 2015).

SYCP1 and ACRBP are members of CTAs families in which the spontaneous immune responses against both of them have been detected in cancer patients (Taherian-Esfahani 2016). SYCP1 interacts with different proteins as a part of the synaptonemal complexes during meiosis and ACRBP participates in spermatogenesis and sperm capacitation (Whitehurst et al. 2010; Costa et al. 2005; Nielsen and Gjerstorff 2016). ACRBP and SYCP1 are located at 12p12–p13 and 1p13.2, respectively (Ono 2001; Xia et al. 2014; Türeci 1998). ACRBP expression has been detected in various types of cancer including bladder (Ono 2001), ovarian (Tammela et al. 2006), and colorectal cancer (Ono 2001).

Also, SYCP1 is expressed at brain (Türeci 1998; Sahin et al. 2000), pancreas (Kubuschok et al. 2004), and hepatobiliary cancers (Luo et al. 2002). Mashino et al. detected high expression of SCYP1 at about 34% of breast cancer patients' tumors (Mashino et al. 2001). Tureci et al. have observed anti-SYCP1 serum reactivity in half of the breast cancer patients (Türeci 1998). Also, many studies have reported high expression of ACRBP in breast tumors (Ono 2001; Whitehurst 2010a, b). Therefore, it seems SYCP1 and ACRBP can be ideal antigens for designing breast cancer vaccine.

The epitope-based vaccines have many advantages over whole-protein antigens immunization, such as enhanced safety, high immunogenicity, and protective potency (Beatty and Gladney 2015; Tang et al. 2015; Gu et al. 2017). Therefore, immunodominant epitopes have long been used to design anti-cancer peptide vaccine (Ebert et al. 2009; Iinuma et al. 2014). Experimental methods for determining the immunodominant epitopes are time-consuming, expensive, and limited. Thus, employing new computational methods for identifying immunodominant epitopes has received lots of attention (Mahdavi et al. 2012; Chen et al. 2011).

Immunological bioinformatics (immunoinformatic) methods are trustable tools for these purposes. They focus on the structure, function, and interactions of the molecules involved in immunity. Many immunoinformatic servers and databases have been introduced for the better understanding of the immune system and its probable responses (Soria-Guerra et al. 2015). Their epitope mapping can identify the T cell epitopes which are determinative in the anti-cancer immune response. This fact has significantly enhanced antigens immunodominant epitopes determination, multiepitope vaccines designing and predicting their immunogenicity and binding affinity for the major histocompatibility complex (MHC) molecules (Fratta et al. 2011; Patronov and Doytchinova 2013). Hence, employing immunoinformatic tools for designing cancer vaccine could be more efficient.

In the present study, immunoinformatic tools were employed for designing novel multiepitope peptide and DNA cancer vaccine consisted of immunodominant fragments of SYCP1 and ACRBP antigens for breast cancer immune therapy.

Methods

Study Design

First, the similar sequences between human and mouse SYCP-1 and ACRBP antigens were identified. The most appropriate regions were selected from the similar candidate sequences according to containing immunodominant epitopes by bioinformatics tools. Subsequently, selected

fragments were linked together by an furin-sensitive linker and the final construct was analyzed at different aspects by different servers. The final construct was analyzed at different aspects by different servers. Reverse translation of the final construct to DNA and adaptation of the DNA sequence to *Escherichia coli* and mouse codon usage were done by bioinformatics servers. A flowchart model was designed to represent the step by step workflow of the study (Fig. 1).

Homology Analysis to Identify Candidate Regions

The SYCP1 and ACRBP amino acid sequences were obtained from NCBI (<http://www.ncbi.nlm.nih.gov>). The similar regions between human and mouse (100% homology) were recognized by Protein Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Then, all the regions were compared

with both mouse and human proteome to recognize the regions without any similarity with both species' proteome (unique regions) for using in the next sections.

Immunoinformatic Analysis

Prediction of MHC I and II Binding Peptides Affinity

Artificial neural network (ANN) and stabilized matrix method (SMM) methods in “IEDB” server (http://tools.immuneepitope.org/analyze/html/mhc_binding.html) (Nezafat et al. 2014) that used IC_{50} [nM] < 50 as the cut off and NetMHCpan 4.0 (<http://www.cbs.dtu.dk/services/NetMHCpan/>) (Vita et al. 2009) were employed to predict MHC-I binding affinity for both human and mouse alleles. The percentile rank < 1% was used. In addition, ProPred-I

Fig. 1 The flow chart of the multi-epitope peptide and DNA vaccines designing

(<http://www.imtech.res.in/raghava/propred1/>) (Singh and Raghava 2003) was employed for confirming the MHC-I binding ability. The maximum accuracy of ProPred-I is 75% at 4% threshold (default threshold). SMM-align, and NN-align, methods in “IEDB” (http://tools.immuneepitope.org/analyze/html/mhc_binding.html) and MHCpred (<http://www.ddg-pharmfac.net/mhcpred/MHCPred/>) (Guan et al. 2003) was employed to recognize MHC-II binding peptides. The default cut-off value of IC_{50} was used. Also, NetMHCIIpan 3.2 (<http://www.cbs.dtu.dk/services/NetMHCIIpan/>) (Jensen 2018) server was used and the threshold for strong binder was set at (% Rank) ≤ 2 . The used reference set of MHC I and II alleles had more than 97% and 99% population coverage in all major ethnicities worldwide, respectively (Greenbaum et al. 2011; Weiskopf 2013; Khalili et al. 2015).

CD8⁺ Cytotoxic T Lymphocytes (CTL) Epitopes Prediction

Three CTL prediction tools including NetCTLpan 1.1 (<http://www.cbs.dtu.dk/services/NetCTLpan/>) (Khalili et al. 2015; Stranzl et al. 2010) with experimental verifications of > 96% to identify new epitopes with 90% likelihood, NetCTL 1.2 (<http://www.cbs.dtu.dk/services/NetCTL/>) (Pandey et al. 2018; Larsen et al. 2007) using all the server provided optional HLA supertypes (threshold for epitope identification > 0.75) and both ANN and SVM prediction approaches of CTLpred (<http://www.imtech.res.in/raghava/ctlpred/>) (Khalili et al. 2015; Bhasin and Raghava 2004). The NetCTLpan 1.1 method integrates prediction of peptide MHC class I binding, proteasomal C terminal cleavage, and TAP transport efficiency. MHC class I binding and proteasomal cleavage is performed using artificial neural networks. TAP transport efficiency is predicted using weight matrix. For NetCTLpan 1.1 percentile Rank < 1% was approved.

Peptide Final Construct

The selected fragments of the antigens were linked together by a furin-sensitive amino acid linker (RVRR). A methionine and a glycine were added to the N-terminal. In addition, His-tag (six histamines) was added to the C-terminal to form the final peptide construct.

Vaccine Features

Prediction of Protein Solubility and Stability

To predict the protein solubility properties, SOLpro (<http://scratch.proteomics.ics.uci.edu/>) (Magnan et al. 2009) which utilizes a two-stage SVM architecture method based on multiple representations of the primary sequence was used. The overall accuracy of SOLpro is estimated over 74% using multiple runs of tenfold cross-validation. In addition, various

physicochemical parameters including amino acid composition, theoretical pI, instability index, in vitro and in vivo half-life, aliphatic index, grand average of hydropathicity (GRAVY), and molecular weight were evaluated by ProtParam online server (<http://web.expasy.org/protparam/>) (Gasteiger et al. 2005).

Proteasomal Cleavage and TAP Transport Prediction

NetChop (<http://tools.iedb.org/netchop/>) and MAPPP (<http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html>) (Subramanian and Chinnappan 2013) were employed for predicting the proteasomal processing. In addition, TAPPred (<http://crdd.osdd.net/raghava/tappred/index.html>) (Sankar et al. 2017) was used to evaluate the binding affinity of the peptides with the TAP transporter.

Tertiary Structure

Homology Modeling and De Novo Structure Prediction

The final construct complete 3D structure was determined by employing Phyer2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) and SWISS-MODEL (<https://swissmodel.expasy.org/interactive>) (Kelley and Sternberg 2009; Guex et al. 2009) which utilize alignment of the target sequence and template structures for homology modeling. For de novo prediction of the 3D structure according to the amino acid sequence, PEP-FOLD (<http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/>) and CABS (<http://biocomp.chem.uw.edu.pl/CABSfold/>) (Maupetit et al. 2009; Blaszczyk et al. 2013) were employed. These servers need the secondary structure for refinement of the tertiary structure of the protein. Therefore, PSIPRED V3.3 (<http://bioinf.cs.ucl.ac.uk/psipredtest>) (EL-Manzalawy et al. 2008) was employed for the prediction of the secondary structure.

Tertiary Structure Refinement

The obtained 3D structure was processed by GalaxyLoop server (<http://galaxy.seoklab.org/>) for generating loop modeling. The loop refining was accomplished by specifying the locations of loop regions in a protein model with ab initio modeling method (Shin et al. 2014; Ko et al. 2012). In the next step, more refinements were done by ReFOLD (<http://www.reading.ac.uk/bioinf/ReFOLD/>) (Shuid et al. 2017; McGuffin et al. 2017), ModRefiner (<https://zhanglab.ccmb.med.umich.edu/ModRefiner/>) and Galaxyrefine (<http://galaxy.seoklab.org/>) (Xu and Zhang 2011) to have the most optimized model.

Tertiary Structure Validation

In order to find the potential errors in initial 3D models, ProSA-web (<https://prosa.services.came.sbg.ac.at/prosa.php>) (Wiederstein and Sippl 2007), PROCHECK program (http://swissmodel.expasy.org/workspace/index.php?func=tools_structureassessment1) (Laskowski et al. 1993) and ERRAT server (<http://nihserver.mbi.ucla.edu/ERRATv2/>) (Colovos and Yeates 1993) were utilized. ProSA-web calculates the overall quality score by analyzing atomic coordination of the model. The ProSA-web z-score is indicated in a plot, which contains the z-score of experimentally determined structures deposited in PDB. The residue-by-residue stereochemical qualities of models were validated by Ramachandran plot obtained from PROCHECK. The ERRAT program evaluates the statistics of non-bonded atom–atom interactions in compared to a database of reliable high-resolution crystallography structures (Kozakov 2010).

B Cell Epitopes Prediction

In order to recognize linear and conformational B cell epitopes, BCPREDS (<http://ailab.ist.psu.edu/bcpred/predict.html>) was employed. BCPred method employs subsequence kernel-based SVM classifier and was trained on homology-reduced dataset of linear B-cell epitopes (with < 80% sequence identity) derived from dataset previously used to test ABCPred. ElliPro (<http://tools.iedb.org/elliPro/>) (Ponomarenko et al. 2008) and DiscoTope (<http://www.cbs.dtu.dk/services/DiscoTope/>) (Kringelum et al. 2012) servers were utilized. Conformational discontinuous B-Cell epitopes were computationally determined by ElliPro tool at IEDB with 0.7 as a minimum Protrusion Index and 6 Å as a maximum distance. The ElliPro tool allowed identification of B-Cell epitopes in a submitted protein three-dimensional structure as a PDB file after homology modeling. ElliPro runs on a database of discontinuous determinants produced from three-dimensional structures of antigen–antibody complexes have shown to have highest accuracy in comparison to six adjacent tools (Ponomarenko et al. 2008). For the DiscoTope serve the default threshold (−3.7) was used for identification of epitopes. In this threshold, the sensitivity and specificity are 0.47 and 0.75, respectively.

Population Coverage

To find out the population coverage of each fragment and the final construct, Epitope/MHC restriction data were loaded at the Population Coverage Calculation tool of IEDB (http://tools.immuneepitope.org/tools/population/iedb_input) (Khalili

et al. 2015). Calculations were set to be performed for MHC class I and II epitopes.

Post-translational Modifications

Post-translational modifications of the DNA vaccine translated peptide including O-glycosylation (<http://www.cbs.dtu.dk/services/NetOGlyc/>) (Steentoft et al. 2013), N-glycosylation (<http://www.cbs.dtu.dk/services/NetNGlyc/>) (Blom et al. 2004), phosphorylation (<http://www.cbs.dtu.dk/services/NetPhos/>) (Blom et al. 1999), and ubiquitination (<http://www.ubpred.org/>) (Radivojac 2010) were predicted. The existence of PEST motifs was analyzed by using PEST-find server (Khalili et al. 2015; Moradi 2017). Protein functional family prediction was performed for the final construct by SVMProt network service based on SVM classification (Khalili et al. 2015).

Data Validation

To evaluate the proper cleavage probability of the proposed epitopes and their availability to the host immune system, retrieval of the original CTL epitopes in the context of final construct was predicted using CTLPred, NetCTL 1.2 and NetCTLpan 1.1, EpiJen, (<http://www.ddgpharmfac.net/epijen/EpiJen/EpiJen.htm>) (Doytchinova et al. 2006), RANKPEP (<http://imed.med.ucm.es/Tools/rankpep.html>) (Reche et al. 2004) and SYFPEITHI (<http://www.syfpeithi.de/>) (Rammensee et al. 1999). The main processing pathway for MHC class I ligands involves degradation of proteins by the proteasome, followed by transport of products by the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER) where peptides are bound by MHC class I molecules and then presented on the cell surface by MHCs. The whole process is modeled using EpiJen. EpiJen is based on quantitative matrices, derived by the additive method and applied successively to select epitopes. In this study, 5% threshold was selected, giving 85% sensitivity (Doytchinova et al. 2006). SYFPEITHI contains more than 7000 peptide sequences of human and other organisms, such as chicken, mouse, and apes, which are known to bind MHC-I and MHC-II molecules (Rammensee et al. 1999). In addition, the final construct population coverage was reinvestigated by the used server at “Vaccine Features” section.

Multiepitope DNA Construct

The template DNA sequence was reverse translated from the peptide sequence and codon optimized by the JCAT (<http://www.jcat.de/>). The JCAT presents a simple method to adapt the Codon Usage to the most of sequenced prokaryotic organisms and selected eukaryotic organisms. The CAI-values were calculated by applying an algorithm from A.

Carbone et al. For more analyzes, GenScript (<https://www.genscript.com/tools/rare-codon-analysis>) was utilized which can calculate GC content and codon frequency distribution (CFD). The CFD is another factor that can affect expression efficiency. (CAI) > 0.8 was selected to support mouse codon adaptation. The GC content among 30–70% was accepted for high-level expression at the desired host. In addition, the Kozak sequence was added to the 5' of the nucleotide sequences to enhance its expression. At last, the DNA construct with suitable restriction enzyme sites (Nhe1, Nco1, and Xho1) at the 5' and 3' ends was designed.

Results

Identification of the Candidate Regions

To design an effective vaccine for both human and mouse due to the necessity of pre-clinical animal experiments before planning human clinical trials, the human and murine amino acid sequences were aligned and only the similar regions (100% homology) were selected (Figs. 2, 3). This will avoid selecting of regions with different sequences

between the two species. The similar regions with appropriate length were identified and listed in Tables S1 and S2. Peptides' appropriate length for MHC-I and MHC-II binding is 8–9 and 12–15 amino acids, respectively. One of the ACRBP regions had similar sequences with some other proteins in the proteome and subsequently was omitted to prevent tolerances. Therefore, just the similar regions with unique sequences in proteome were selected as candidate regions for immunoinformatic analysis (Tables S1, S2).

Selection of the Most Immunogenic Regions of Antigens

The (RCQHKIAEMVALMEKHKHQYDKI) region from SYCP1 isoform 1 and (YIQYPNYCSFKSQCL) region from ACRBP were selected as the most immunogenic regions. These regions contained multiple epitopes with high affinity for binding to several MHCs. Their epitopes with the high binding affinity (IC₅₀ values < 50 nM or confirmed with ProPred-I) are listed in Tables S3 and S4. The average world population coverage of the SYCP1 and ACRBP selected fragments were about 80% and 71%, respectively (Table S5). CTL epitopes are so important

Human	1	MRKPAAGFLPSLLKVLPLAPAAAQDSTQASTPGSPLSPTEYERFFALLTPTWKAETTC	60
		M AAGFL LL+VLLLP P +A++S ASTPGSPLS TEYERFFALLTPTWKAETTC	
Mouse	1	MMNLAAGFLMLLLEVLLLPGLTPLSAEESP-ASTPGSPLSSTEYERFFALLTPTWKAETTC	59
Human	61	RLRATHGCRNPTLVQLDQYENHGLVPDGAVCNLPYASWFESFCQFTHYRCSNHVYAKR	120
		RLRATHGCRNPTLVQLDQYENHGLVPDGAVCNLPYASWFESFCQF YRCSNHVYAKR	
Mouse	60	RLRATHGCRNPTLVQLDQYENHGLVPDGAVCNLPYASWFESFCQFAQYRCSNHVYAKR	119
Human	121	VLCSQPVSIPLSNTLKEIEASAEVSPTMTSPISPHFTVTERQTQFPWPERLSNNVEELL	180
		CSQPVSIPLSNTLKE+E+SAEV PT+MT+PI H T TE Q FQWPERL+NNVEELL	
Mouse	120	LRCSQPVSIPLSNTLKEVESSAEVPTMTPTIVSHATATEHQAFQFPWPERLNNVEELL	179
Human	181	QSSLSLGGQEQAPEHKQEQGVEHRQEPTEQEHK-QEEGQKQEEQEEQEEGKQEEGQGTK	239
		QSSLSLGG++Q + Q +H+QE QEHK +E +++E+EEE+EEE KQEEGQGT+	
Mouse	180	QSSLSLGGKDQSSRRPGQE-QHKQEQIQEHKLEEAQEQEEQEEEEEEAKQEEGQGTG	238
Human	240	EGREAVSQLQTDSEPKFHSESLSSNPSSFAPRVREVESTPMIMENIQELIRSAQEIDEMN	299
		G E+VS+LQ+DSEPKF S+SLSSNPS F PRVREVES P++M+NIQELIRSAQE+DEM	
Mouse	239	AGLESVSRQLQSDSEPKFQSQSLSSNPSFFTPTPRVREVESAPIMMKNIQELIRSAQEMDEM	298
Human	300	EIYDENSYWRNQNPGSLQLPHTTEALLVLCYSIVENTCIIPTAKAWKYEMEEILGFGKS	359
		E+YD++ WR+Q+ GSL QLP H E L+VLCYSI+ENTC +TPTAKAW YEMEEILGFG S	
Mouse	299	ELYDDS--WRSQSTGSLQQLPHMETLMVLCYSIVENTCMTPTAKAWSYEMEEILGFGDS	356
Human	360	VCDLGRHRMSTCALCDFCSLKLEQCHSEASLQRQCDTSHKTPFVSPLLASQSLSIGNQ	419
		VCD+LGRHR + C LC FCSLKLEQCHSEAS+ RQ+CD SHK PF+SPLL++QS+S GNQ	
Mouse	357	VCDNLGRHRHTAACPLCAFCSLKLEQCHSEASVVRQKCDASHKIPFISPLLSAQSIISTGNQ	416
Human	420	VGPESGRFYGLDLYGGLHMDFWCARLATKGCEDVRVSGWLQTEFLSFQDGDFTKICDT	479
		P+ GRF GL++YGG L +FWC RLA KGCED RVS WL+ EFLSFQ+GDFPTKICDT	
Mouse	417	ARIPDKGRFAGLEMYGGLSSEFWCNRLAMKGCEDDRVSNWLKAEFLSFQEGDFTKICDT	476
Human	480	DYIQYPNYCSFKSQCLMRNRNRKVSRRMCLQNETYSALSPGKSEDEVVLRWSQEFSTLTL	539
		+YIQYPNYCSFKSQCL+RN+NRK+SRMRC+ NE Y+ LS KSE+V+LRWSQEFSTL +	
Mouse	477	NYIQYPNYCSFKSQCLLRNQNRKMSRMRCLNERYNVLSLAKSEEVLIRWSQEFSTLAI	536
Human	540	GQFG 543	
		GQFG	
Mouse	537	GQFG 540	

Fig. 2 Alignment of murine and human ACRBP antigens' amino acid sequences. The regions which has 100% similarity between human and mouse (candidate regions) are highlighted

Human	1	MEKQKPFALFVPPRSSSSQVSAVKPQTLGGDSTFFKSFNKCTEDDFEFPPFAKTNLSKNGE	60
		MEKQKPF LFVPPR SSSQVSAVKPQT GGDS +FK+ NKCTE DF PF ++ E	
Mouse	1	MEKQKPFLLFVPPRLSSSQVSAVKPQTAGGDSNYFKTVNKCTEGDFGVFPFTMSS---RE	56
Human	61	NIDSDPALQKVNFLPVLEQVGNSD-CHYQEGKDSLENSEGLSRVYSKLYKEAEKIKKW	119
		NID DPA QK++ LP+LEQV NS CHYQEG+ DSD ENSE +SR+YSKLYKEAEKI KW	
Mouse	57	NIDKDPAFQKLSILPMLQVANSCHYQEGVNDSDFENSEPMSRLYSKLYKEAEKIQKW	116
Human	120	KVSTAEALRQKESKLQENRKII EAQRKAIQELQFGNEKVS LKLEEGIQENKDLIKENNAT	179
		KVS E+EL+QKE+KLQENRKII+AQRKAIQELQF NEKVS LKLEE IQENKDLIKENNAT	
Mouse	117	KVSIESELKQKENKLQENRKIIQAQRKAIQELQFENEKVS LKLEEEIQENKDLIKENNAT	176
Human	180	RHLNLLKETCARSAEKT KKYEYEREETRQVYMDLNNIEKMITAFEELRVQAENSRLEM	239
		H CNLLKETCARSAEKT KYEYEREETRQVY +DLN+NIEKMI AFEELRVQAEN+RLEM	
Mouse	177	IHWCNLLKETCARSAEKT NKYEYEREETRQVYVDLNSNIEKMILAFEELRVQAENARLEM	236
Human	240	HFKLKEDYEKIQHLEQEYKKEINDKEKQVSLLLIQITEKENMKMDLTFLLEESRDKNVQL	299
		HFKLKED+EKIQHLE+EY+KE+N+KE QVS LLIQ EKENMKMDLTFLLEESRDK NQL	
Mouse	237	HFKLKEDHEKIQHLEEEYQKEVNNKENQVSELLIQSAEKENMKMDLTFLLEESRDKANQL	296
Human	300	EEKTKLQSENKQSI EKQHHLTKELEDIKVSLQRSVSTQKALEEDLQIATKTICQLTEEK	359
		EEKTKLQ ENLK+ EK+ HLT ELEDIK+S+QRS+ STQKALEEDLQIATKTI QLTE K	
Mouse	297	EEKTKLQDENLKESEKDHILTSELEDIKMSMQRSMTQKALEEDLQIATKTISQLTEVK	356
Human	360	ETQMEESNKARAAHS FVTEFETTVCSLEELLRT EQQRLEKNEDQLKILTMELQKKSSEL	419
		E QMEE NKA+ HSFVTE + T C+ LEELLRT EQQRLEKNEDQLK ++T+ELQKKS+EL	
Mouse	357	EAQMEELNKA KTTTHS FVTELKATCTLEELLRT EQQRLEKNEDQLKLITVELQKKSNE	416
Human	420	EEMTKLTNNKEVELEELKVLGEKETLLYENKQFEKIAEELKGTEQELIGLLQAREKEVH	479
		EEMTK NNKEVELEELK +L E + LL E KQ EK+AEEL+ EQEL LL+ REKEVH	
Mouse	417	EEMTKFKNKEVELEELKNILAEDQKLLDEKKQVEKLAEELEKEQEQLTFLLETREKEVH	476
Human	480	DLEIQLTAITTS EQYYSKEVKDLKTELENEKLKNTELTSHCNKLSLENKELTQETS DMTL	539
		DL+ Q+T TSEQ+Y K+V+++KTELE EKLKNTELT+ C+ L LENK+ QE SDM L	
Mouse	477	DLQEQT VTKTSEQHYLKQVEEMKTELEKEKLKNTELTASCDMLLLENKKFVQEASDMAL	536
Human	540	ELKNQ QEDINNNKKQEERMLKQIENLQETETQLRNELEYVREELKQKRDEVCKLKDSEE	599
		ELK QEDI N KKQEER+LKQIENL+E E LR+ELE VR+E Q+ DEVCKLKDSEE	
Mouse	537	ELKKHQEDIINCKKQEERLLKQIENLEEKEMHLRDELESVRKEFIQQGDEVCKLKDSEE	596
Human	600	N-----CNLNRKQVENKNKYIEELQQENKALKKKKGTAESKQLNV	638
		N CNL+KQVENK+K IEEL QENK LKKK +AE KQLN	
Mouse	597	NARSIECEVLKKEKQMKILESCKNNLKKQVENKSKNIEELHQENKTLKKKSSAEIKQLNA	656
Human	639	YEIKVNKLELELES AKQKFGEITDTYQKEIEDKKISEENLLEEVEKAKVIADAEAVKLQKE	698
		YEIKV+KLELELES KQ+F E+T+ YQKEIE+KKISE LL EVEKAK DEAVKLQKE	
Mouse	657	YEIKVSKLELELESTKQRFEEMTNNYQKEIENKKISEGKLLGEVEKAKATVDEAVKLQKE	716
Human	699	IDKRCQH KIAEMVALMEKHKHQYDKIIEERDSELGLYKSKEQEQQSSLRASLEIELSNLKA	758
		ID RCQH KIAEMVALMEKHKHQYDKI +EERDSELGLYK++EQEQSS + +LE ELSN++	
Mouse	717	IDLRCQH KIAEMVALMEKHKHQYDKIVEERDSELGLYKNREQEQQSSAKIALETLSNIRN	776
Human	759	ELLSVKKQLEIEREEKEKLKREAKENTATLKEKKDKKTQTFLLETPE-IYWKLD SKAVPS	817
		EL+S+KKQ +E+++ + AKENTA LK+KKDKK Q LLE+PE WK DSK PS	
Mouse	777	ELVSLKKQ-LEIEKEEKEKLKMAKENTAILKDKKDKKI QASLLESPEATSWKFDSKTTTPS	835
Human	818	QTVSRNFTSVDHGISKDRDYLWTS AKNTLSTPLPKAYTVKTPPKLQQREN LNPIIEE	877
		Q +SR +S+D G SKD RD L SAK+ L T + K YTVKTPK + QREN IP	
Mouse	836	QNISRLSSMSD SGSKDNDRNLRASAKSILPTTVTKEYTVKTPPKSIYQRENKYIPTGG	895
Human	878	SKKKRKMAFEFDINS DSSETD LLSMVSEETLKTLYRNPPASHLCVKT PKKAPSSLT	937
		S KKRK AFEFD+NSDSSET DLLS+VSEE+ LY +NNPP SHL VKTPK+ P SL+	
Mouse	896	SNKKRKTAFEFDVNS DSSETADLLS LVSEEDVSNRLY-DNNPPD SHLLVKT PKQTPLSLS	954
Human	938	TPGSTLKFGAIRKMREDRWAVIAKMDRKKKLKEAEKLF	975
		TP S +KFG+++KMREDRW IAK+DRK++LKEAEKLF	
Mouse	955	TPASFMKFGSLKKMREDRWTTIAKIDRKRRLKEAEKLF	992

Fig. 3 Alignment of murine and human SYCP1 antigens' amino acid sequences. The candidate regions are highlighted

in cancer vaccine efficiency due to T lymphocytes critical role in the immune system anti-cancer response. Both fragments had multiple CTL epitopes which are listed in Tables S6 and S7.

Final Construct of the Multiepitope Peptide Vaccine

The final construct of the multiepitope peptide vaccine which had 51 amino acids length is schematically illustrated

Fig. 4 Schematic diagram of the multi-epitope vaccine amino acid sequence consists of methionine, glycine, SYCP1 region, furin-sensitive linker, ACRBP region, and His-tag

in Fig. 4. The construct is consisted of the two selected fragments which were fused to each other by the furin-sensitive linker. The methionine was added at the N-terminal to form the AUG start code.

The Multi-epitope Peptide Vaccine Features

Solubility and Stability

Peptides physio-chemical parameters have considerable effects on their properties like stability, transportation, and immunogenicity (Goodwin et al. 2012). The molecular weight (Mw) for the multi-epitope peptide vaccine was 6.38 kDa and the theoretical isoelectric point value (pI) was computed 9.61. In addition, the peptide solubility tendency upon overexpression in *E. coli* was 0.9. Instability index predicts whether a protein is stable or unstable, if the predicted instability index of a protein is below 40 then it's called stable and proteins with the value above than 40 are comes under the category of unstable proteins. The instability index was computed 33.38 for the multi-epitope peptide vaccine which categorizes as a stable protein. The estimated half-life in mammalian reticulocytes, yeast, and *E. coli* was about 30 h, 20 h, and more than 10 h, respectively. The grand average hydropathicity (GRAVY) and aliphatic indexes were defined -1.127 and 53.53 , respectively. Negative value of GRAVY for the multi-epitope peptide vaccine represents the hydrophilic nature of the peptide. It should be mentioned the peptides with positive value of GRAVY are hydrophobic. The estimated value of aliphatic index represents the thermostable nature of the multi-epitope peptide vaccine and as the value of aliphatic index increases, the peptide exhibits greater thermo stability (Ikai 1980; Kyte and Doolittle 1982).

Proteolysis and TAP Transport

Proteolysis and transporter associated antigen presentation (TAP) are important for processing of the MHC-I and forming peptide-MHC-I complexes. The cleavage site on the multi-epitope peptide vaccine was evaluated by NetChop and MAPPP servers. The number of possible predicted cleavage sites by NetChop and MAPPP servers were 12 and 16, respectively (Fig. 5). The prediction of the TAP binding

Fig. 5 The predicted proteasome cleavage sites of the multi-epitope peptide vaccine. The positive predictions (cleavage sites) are displayed in green while below the threshold value (0.5) predicted regions are in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article). (Color figure online)

affinity of the multi-epitope peptide was achieved using TAP-Pred server. The results showed three segments with high binding affinity and ten segments with intermediate binding affinity to TAP protein. The high-affinity peptides are illustrated in Table S8.

Determining The 3D Structure of The Multi-epitope Peptide Vaccine

The 3D structure is necessary for determining the conformational B cells epitope by Ellipro server. Therefore, phyre2 and SWISS-MODEL servers were employed to determine the peptide vaccine 3D structure through homology modeling. However, the alignment quality of the query with the template models was only 47% (low confidence). Due to homology modeling failure, the PEP-FOLD and CABS-fold servers were used for de novo prediction of the 3D structure (Manijeh et al. 2013). The secondary structure is a prerequisite factor for de novo 3D structure prediction.

The obtained 3D structure was refined by GalaxyLoop, ReFOLD, and ModRefiner servers (Fig. 6). The results were submitted to ERRAT and ProSA-web to verify the quality

Fig. 6 The predicted tertiary structure of the multi-epitope peptide vaccine. **a** The initial model. **b** The refined model after precise loop refinement and energy minimization. **c** The initial and refined 3D models superimposing

Fig. 7 z-Score plot for 3D structure of the construct. The z-score of **a** the initial model is -2.57 . **b** The z-score of the model after refinement processes is -3.22 . z-Score plot contains z-Scores of all experimentally protein chains in PDB determined by NMR spectroscopy (dark blue) and X-ray crystallography (light blue). The plot shows

results with a z-Score -10 . The z-Score of the peptide vaccine is presented in large black dot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article). (Color figure online)

and potential errors in a crude 3D model. The best primary model z-score and overall quality factor were -2.57 and 74.41 for ProSA-web and ERRAT, respectively.

The 3D structure refinement was done and the model parameters turned to -3.22 and 100 (Figs. 7, 8).

For final validation of the refined 3D structures, Ramachandran maps were performed for primary and the refined model by PROCHECK server (Fig. 9). Before refinement, 76% of residues were located in the favorite regions according to Ramachandran plot and after the refinement, this amount changed to 91% . All related data

Fig. 8 The overall quality factor plot (ERRAT) of **a** the initial model is 74.41% and **b** the final model after refinement processes is 100%. In ERRAT plot, regions of the 3D model that can be rejected at the 95% confidence level are shown in gray lines and regions that can be rejected at the 99% level are depicted in black lines. The overall quality factor manifests the percentage of protein for which the computed error value falls under the 95% rejection limit. Good high-resolution structures usually generate values about 95% or higher

of the primary and the refined models are presented in Table S9.

Determining the B Cells Epitopes

Many studies support the critical role of B cells in anti-tumor immune response (Tsou et al. 2016). Therefore, the final refined 3D structure was applied as an input in ElliPro and DiscoTope servers for identifying conformational B cells epitopes (Table S10 and Fig. 10). In addition, Identification of the B cell linear epitopes was carried out by BCPRED and ElliPro servers. The multiepitope peptide vaccine had 4 linear and 6 conformational B cells epitopes (Table S11).

Post-translation Modifications

One of the most determining factors in designing DNA vaccines is post-translational modification of its translated peptide. Post-translational modifications can significantly affect stability and intracellular processing of a peptide for MHC pathway. The multi-epitope peptide vaccine length was 51 amino acids (6.35 kDa) without any PEST site. The translated peptide does not have any function according to predictions of the utilized servers. All the predicted ubiquitination, phosphorylation, and glycosylation sites are illustrated in

Fig. 9 Validation of the peptide vaccine 3D model using Ramachandran plot. The Ramachandran plots of **a** the initial model indicated that 76.6% of residues are located in most-favored regions (a, b and l), 14.9% in additional allowed regions (a, b, l, and p), 6.4% in generously allowed regions (~a, ~b, ~l, ~p) and 2.1% in disallowed regions of the plot and **b** the model after refinement revealed that 91.5% of residues are located in most-favored regions, 6.4% in additional allowed regions, 2.1% in generously allowed regions and

only 0% in disallowed regions. Favored and the most-favored regions demonstrated with yellow and red colors, respectively. The generously allowed regions are indicated in pale yellow, and the disallowed regions are in white color. Residues of protein are shown in black squares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article). (Color figure online)

Fig. 10 The predicted conformational B cell epitopes from the 3D structure template are exhibited in **a–c** ball and stick model. (The residues of predicted epitopes are simulated by yellow balls and white sticks are for non-epitope and core residues). (Color figure online)

Fig. 11 The predicted ubiquitination and phosphorylation cited at the final construct after post-translational modifications. Ubiquitination and phosphorylation sites are illustrated as blue and yellow beads.

The green beads exhibit sites which underwent both phosphorylation and O-glycosylation. *K* lysine; *S* serine; and *Y* tyrosine. (Color figure online)

Fig. 11. No N-glycosylation site was predicted for the peptide vaccine. However, two sites of O-glycosylation including the 38 and 41 residues were predicted.

Data Validation

Due to the importance of the CTLs at cancer immune therapy, intensive CTL epitopes validation was done with different servers. Most of the reassigned CTLs epitopes within the final construct were the same as the epitopes assigned in the first place. As Table S12 illustrates, the peptide vaccine has multiple CTL epitopes with MHC-I binding ability. In addition, the predicted population coverage worldwide for the final construct according to the ability for binding to MHC alleles was 90% (Table S5).

The Multiepitope DNA Vaccine Development and In Silico Cloning

Codon adaptation is a way to attain major expression rate for foreign genes. Especially, when the host codon usage differs from that gene originated organism. Therefore, the

final construct was reverse translated and codon optimized. The optimized codon adaptation index (CAI) for mouse and bacteria were 0.92 and 0.87, respectively and the overall GC content was 52.54% (Fig. 12).

The final construct contained suitable restriction enzymes sites (Fig. 13) and Kozak sequences in its structure. These restriction sites cause ability for cloning into pcDNA3.1 to form a multiepitope DNA vaccine or pET-28a for express at *E. coli*.

Discussion

Cancer is one of the leading cause of death in human societies (Stewart 2014). In spite of significant developments in the knowledge about cancer, the current treatments outcomes are not satisfying. Recently, cancer immune therapy has received a lot of attention due to considerable effectiveness and low side effects (Urruticoechea et al. 2010). It has different approaches including cell-mediated immune therapy, monoclonal antibodies, and vaccines (Regan et al. 2016; Lin and Li 2013).

E

GCTAGCACCATGGGCCGCTGCCAGCACAAAATCGCCGAAATGG
 TGGCCCTGATGGAAAAACACAAACACCAGTACGACAAAATCCG
 CGTGCGCCGCTACATCCAGTACCCAAACTACTGCAGCTTCAAAA
 GCCAGCAGTGCCTGCACCACCACCACCACCTGACTCGAG

Fig. 12 The Codon adaptation index value and the codon frequency distribution (CFD) of the DNA sequence. **a** The CAI value is 0.92 for the murine host. **b** The codon with frequency distribution of 91–100, 81–90, 71–80 and 61–70 are respectively 78%, 7%, 0% and 13% in gene sequence for murine host. **c** The CAI value is 0.87 for *E. coli*. **d** codon with frequency distribution of 91–100, 81–90, 71–80, 61–70,

51–60 and 41–50 are respectively 58%, 0%, 29%, 7%, 1% and 1% for *E. coli* host. (The CAI equal 1 and over 0.8 are announced as perfect and good for expression in the chosen host, respectively and codons with values lower than 30 can prevent efficient expression). **e** The optimized DNA sequence of the final construct

Fig. 13 The schematic diagram of the sequence of the constructed DNA vaccine. It contains NheI and NcoI restriction enzymes sequences at 5' end and also an XhoI sequence at 3' ends. A furin-

sensitive linker sequence fuses the two epitopes to each other. Also, a Kozak sequence is designed to enhance transcription

Cancer vaccines have exhibited considerable advantages in clinical trials (Finn 2014). The main purpose of vaccinations therapy is the efficient presentation of appropriate tumor-specific antigens to the patients' immune system. The most suitable antigens for cancer vaccination therapy are TAAs subtypes like CTAs (Vinay et al. 2015; Vigneron 2015). The CTAs expression in normal tissue is extremely rare and malignant transformations cause their overexpression in the cancerous cells (Salmaninejad et al. 2016; Cheng and Mruk 2012). Therefore, CTAs have been introduced as appropriate targets for cancer vaccination and anti-cancer immune response. Many clinical trials based on CTAs vaccines have been completed like MAGE and NYESO antigens vaccine therapy for melanoma patients which had significant outcomes (Fratta et al. 2011).

This is apparent that targeting multiple immunodominant epitopes from different antigens can enhance the vaccination outcome (Buonaguro et al. 2011). Therefore, immunodominant epitopes have long been used to design peptides for vaccination against cancer (Ebert et al. 2009; Iinuma et al. 2014). Immunoinformatic tools and databases are getting more involved in designing novel cancer vaccines. They are one of the main methods for the selection of antigens immunodominant epitopes (Backert and Kohlbacher 2015).

In this study, two CTAs including SYCP1 and ACRBP were utilized for designing a multiepitope DNA and peptide cancer vaccine by bioinformatic tools. SYCP1 and ACRBP expression and up-regulation have been observed in many cancers. Therefore, a peptide vaccine consists of their immunodominant epitopes can exhibit high coverage for a vast variety of cancers. In cancer patients, activation of both cellular and humoral immune response against SYCP1 and ACRBP have been reported which demonstrate immunogenicity of these antigens in the autologous host (Taheirani-Esfahani 2016). Cancer vaccines should be assessed by animal experiments before intervening clinical trials. This fact is more prominent in cancer which murine tumor models are using vastly (Martin et al. 1986). Therefore, the regions with completely the same amino acid sequences between human and mouse were selected. This can make it possible for applying the animal experiments to human. Selection of epitopes from human segments without considering the murine epitopes may have some disadvantages. A selected immunodominant epitope for human segments may not have the same amino acid residue in comparison with murine. The murine cancer cells may exhibit the antigen with differences in the selected epitope residue. Therefore, it wouldn't be recognized by the immune system which has been provoked against human epitopes. It would be a big sight for an *in silico* analysis to consider both the mouse and human at the same time for selecting the immunodominant epitopes. This will make the result of *in vivo* experiments of the designed cancer vaccine more trustworthy.

In part of cancer vaccination, immune tolerance is a big challenge while utilizing autologous antigens and this problem can be solved by comparison of the antigens sequences with the proteome to select the unique epitopes (Makkouk and Weiner 2015). In this study, the selected antigens regions were compared with both human and mouse proteomes to avoid immune tolerance in both species (Tables S1, S2).

In cellular immune response against cancer, CTLs play the most important role in attacking cancer cells by producing interferon gamma and tumor cells apoptosis induction. Accordingly, CTLs have been shown to associate with better survival for many cancer patients (Kessler and Melief 2007; Chen et al. 2014). Although CTLs have been considered as the main player in anti-tumor response resulting from vaccines, several studies have suggested that the CD4⁺ T cells orchestrate a broad range of immune responses against cancer. This fact is more prominent at peptide vaccination therapy. This cell population differentiates into multiple sublineages which can induce and maintain anti-tumor immune responses (Galaine et al. 2015; Hadrup et al. 2013). Due to the undeniable function of cellular immunity at tumor eradication; at first, the T cell epitopes which are associated with MHC-I and II binding ability were selected. The two selected regions contained different epitopes for binding to a broad spectrum of HLAs (Tables S3, S4). Restricted binding to specific HLAs could merely be effective in a population with a high frequency of these HLAs (Imanishi 1992). However, restricted binding to specific HLAs limits the coverage to a specific ethnicity because of the dramatic polymorphism of the HLA genes and different expression frequencies in each ethnicity (Khalili et al. 2015). The multiepitope cancer vaccine had about 90% HLA coverage in worldwide (Table S5) which can be related to containing multiple epitopes from two different CTAs (Bui et al. 2006).

The final construct of multiepitope peptide vaccine was formed by linking the two selected fragments from SYCP1 and ACRBP together with a furin-sensitive linker (Fig. 4). This linker can enhance the CTL role in the immune response. It causes effective processing of the multiepitope cancer vaccine in the trans-Golgi network and endoplasmic reticulum. Therefore, the furin-sensitive linkers are necessary for effective fragments separation (Lu et al. 2004). In addition, the final construct exhibited appropriate immunogenicity for B cells. B cells ability to shape the other immune cells functions by secreting cytokines, providing co-stimulation, and especially presenting antigens has made them important in cancer vaccine therapy. The B cells epitopes improve the peptide vaccine uptake by the B cells, presentation to the CD4 lymphocytes, and subsequent CD8⁺ cells activation (Tsou et al. 2016; Martin and Chan 2006). Therefore, peptide vaccines should contain not only CTL epitopes but also B cells epitopes. The B cells epitopes can be divided into linear and conformational. The multiepitope

peptide vaccine contained three conformational epitopes in its 3D structure and four linear epitopes (Tables S10, S11).

Many post-translational modifications (PTM) have been introduced for proteins. Some of them like ubiquitination, phosphorylation, and glycosylation can significantly affect the cancer vaccines efficiency. Ubiquitination sites with peptide vaccines facilitate their proper targeting to proteasome, degradation, and epitopes presentation. Therefore, ubiquitination improves the antigen entry into the MHC-I pathway and enhances the CD8⁺ T cell responses (Rodriguez et al. 1998; Leifert et al. 2004). Phosphorylation and glycosylation are two others important PTMs. Phosphorylated antigens could better undergo ubiquitination and proteasomal degradation (Ang and Harper 2005). There are six predicted phosphorylation sites within the multiepitope peptide vaccine which could function as a degradation signal (Zarling et al. 2000; Dougan et al. 2012). The impact of glycosylation on immunogenicity and antigenicity have been mentioned in many studies. Especially, the O-glycosylation sites which cause the epitopes display by both MHC classes (Zarling et al. 2000). PTMs analysis revealed 2 glycosylation sites in the peptide. In general, our peptide vaccine exhibits appropriate PTMs which enhance its immunogenicity and efficacy (Fig. 11).

The multiepitope peptide vaccine has appropriate stability and solubility. Stability and solubility are critical issues in both translation and post-translation. High solubility not only supports stability but also enhances bioavailability, immunogenicity, and decreases undesired side effects (Prasad et al. 2011; Vazquez et al. 2011). It had proteasome cleavage sites and TAP transport affinity which cause epitopes transport to the endoplasmic reticulum to form peptide-MHC complexes. It could provoke CTLs because of its multiple CTL epitopes. The main concern after formation of final construct is to disrupt the initial epitopes of antigens due to manipulations like adding linkers and etc. Therefore, Different servers including SYFPEITHI were employed to validate the final construct according to CTLs binding affinity. The data validation demonstrated that the modifications and manipulations couldn't change the primary epitopes (Table S12). The CTL epitopes were validated by NetCTLpan and NetCTL which integrate MHC-I-binding ability, proteasomal C terminal cleavage, and TAP transport efficiency to identify restricted CTL epitopes.

The main purpose of the final construct was to form multiepitope DNA and peptide vaccines. The final structure was reverse translated and codon optimized for both bacteria and mammals. Therefore, the final DNA construct would gain the ability for integrating into the pcDNA3.1 vector as DNA vaccine and/or into pET-28a vector for producing the peptide vaccine in bacteria expression system. For the enhancement of the translation process, the Kozak sequence (ACCATG

G) was added to 5' of the DNA construct (Kozak 1987). Its ATG was used to form the start codon (AUG which translates to methionine) in mRNA sequence (Drabkin and RajBhandary 1998). In addition, the GC nucleotides were added to the last nucleotide of the Kozak sequence (G) to form the GGC codon for avoiding from the frameshift (Fig. 10). This codon will be translated to Glycine.

Conclusion

In this study, a novel peptide vaccine was designed consist of SYCP1 and ACRBP immunodominant CTLs and B cells epitopes for breast cancer immunotherapy. The multiepitope vaccine had about 90% HLA coverage in worldwide which can be related to containing multiple epitopes from two different CTAs. Therefore, the multiepitope peptide and DNA vaccines efficacy should be evaluated by future *in vivo* experimental studies.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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