

In silico Evaluation of PLAC1-fliC As a Chimeric Vaccine against Breast Cancer

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ABSTRACT

Background: Breast cancer is one of the most prevalent cancers among women. Common cancer treatment methods are not effective enough, and there is a need for a more efficient treatment procedure. Cancer vaccine is a novel immunotherapy method that stimulates humoral and/or cellular immunity against cancer. PLAC1 is a cancer/testis antigen, prevalent in breast cancer and rarely found in normal tissues. FliC, as a bacterial adjuvant, when fused to PLAC1 can elicit humoral and cellular responses. Therefore, PLAC1-fliC is a chimeric protein, which can be considered a suitable candidate against breast cancer. **Methods:** ProtParam was used to evaluate the physicochemical properties of PLAC1-fliC. Second structures were determined using the GOR V server. PLAC1-fliC 3D structure was modeled by Phyre2, and it was refined using GalaxyWEB. The refined model was submitted to RAMPAGE, PROCHECK, and ProSA-web for validation. Antigenicity and allergenicity of the construct were predicted by ANTIGENpro, VaxiJen, AllergenFP, and SDAP databases. Then MHC-I- and MHC-II-binding epitopes of PLAC1-fliC were forecasted by NetMHC 4.0 and NetMHCII 2.3 Servers. Finally, Ellipro and CTLpred were employed to predict B-cell and CTL epitopes. **Results:** The construct was evaluated as a stable fusion protein, which could be antigenic and could stimulate B and T cells against breast cancer. **Conclusion:** PLAC1-fliC, as a cancer vaccine candidate, might be suitable and specific for breast cancer, which could evoke humoral and cellular immunity against this type of tumor. **DOI: 10.29252/ibj.24.3.173**

Keywords: Bioinformatics, Breast cancer, Cancer vaccines, PLAC1

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INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and cancer-related death amongst women. Annually, more than two million breast cancer cases are reported worldwide^[1]. Despite the conventional therapeutic modalities for breast cancer treatment, these therapies have been suggested

to be rather ineffective^[2].

New studies have shown that immunotherapy can be an effective and alternative choice in treating breast cancer^[3]. Immunotherapy consists of anti-cancer antibody, cancer vaccine, and T-cell receptor engineering^[2]. PLAC1 is a cancer/testis antigen with a significant role in cancer progression and invasion^[4]. Cancer/testis antigens are specific antigens expressed

List of Abbreviations:

CTL, cytotoxic T lymphocyte; PLAC1, placenta-specific protein 1; TLR, toll-like receptor; 3D, three-dimensional

in germ-line cells like testis, fetal ovary, and placenta. These antigens are also expressed in some cancer cells, but rarely found in normal tissues^[5]. Studies have revealed that PLAC1 expression in normal cells is not adequate to be measured, while its expression in cancer cells is measurable^[5,6]. Recently, it has been displayed that PLAC1 plays a critical role in tumor invasion and metastasis through Furin/NICD/PTEN/AKT axis^[7]. PLAC1 is a type II membrane bound protein, which its 5-22 amino acids form a transmembrane helix, and has a large extracellular domain consisting of 23-212 amino acids. A truncated zona pellucida domain in the extracellular part of PLAC1 is made of amino acids 29-119^[8]. New research on PLAC1 has indicated that it is well expressed in a versatile cancer cases, specifically in breast cancer, but not expressed in normal tissues, except testis^[9]. Therefore, PLAC1 could be a specific target for breast cancer immunotherapy.

Vaccines need an adjuvant to be more effective^[10]. Adjuvants induce TLR, which results in helper T-cell activation. Bacterial flagellin is one of the most important protein adjuvants that induces TLR5 receptors^[11]. FliC protein is the main component of flagellin and encoded by *fliC* gene^[12]. *Salmonella enterica* serovar typhimurium FliC, as an efficient adjuvant, is widely used in vaccine research^[13]. FliC is made of four domains, including D0, D1, D2, and D3. D1 is responsible for TLR5 binding and dimerization of TLR5s and triggers the downstream signaling and stimulates cells to secrete proinflammatory cytokines such as TNF- α ^[14]. Flagellin is a TLR5 binding ligand and starts downstream signaling through MyD88 pathway, which activates innate immunity. It has been shown that the innate immune system motivation results in cytokines secretion and dendritic cells activation^[15].

The aim of this study was to design a fusion protein construct, as an effective vaccine, consisting of PLAC1 (as a specific antigen) and *Salmonella enterica* fliC (as a bacterial adjuvant) that can stimulate humoral and cellular immune responses against breast cancer. This construct was evaluated using bioinformatics online web servers.

MATERIALS AND METHODS

Construct design

In this study, the amino acid sequences of PLAC1 and fliC were extracted from Uniprot database (<https://www.uniprot.org/>) in FASTA format (Uniprot id: Q9HBJ0). Amino acids 23-212 of PLAC1 were considered for the construct design, and residues 1-22 were neglected because they are located in plasma

membrane and cytosol, and the humoral immunity does not have access to them. A flexible linker (GSGGSGGSGGSG) was located between PLAC1 antigen and fliC adjuvant. Our final construct was PLAC1 (23-212)-linker (GSGGSGGSGGSG)-fliC.

Prediction of physicochemical properties and secondary structure

To predict different physicochemical features, such as instability index, isoelectric point, aliphatic index, grand average of hydropathicity, and molecular weight for PLAC1-fliC, we utilized ProtParam server (<https://web.expasy.org/protparam/>)^[16]. Secondary structure of PLAC1, fliC, and PLAC1-fliC were predicted using GOR V server (<https://abs.cit.nih.gov/gor/>) and were compared together^[17].

Tertiary structure prediction and refinement

Phyre2 server (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>) was employed to predict the 3D structure of the construct based on homology modeling method^[18]. The model was refined using GalaxyRefine server (<http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE>)^[19], and the best refined model was selected and submitted for next steps.

Validation of tertiary structure

For the validation of the model, the following servers were used: RAMPAGE (<http://servicesn.mbi.ucla.edu/PROCHECK/>), ProSA-web (<https://prosa.services.sbg.ac.at/prosa.php>), and PROCHECK (<http://servicesn.mbi.ucla.edu/PROCHECK/>). RAMPAGE server has ability to check stereochemical qualities of the models peptide bonds and shows the number of residues in favored, allowed and outer areas in a Ramachandran plot. ProSA-web has a diagnostic method that is able to analyze protein structures based on all the available protein structures^[20]. PROCHECK server was employed for evaluating the stereochemical quality of the PLAC1-fliC^[21]. The results of all the three servers were compared before and after 3D structure model refinement.

Antigenicity and allergenicity prediction

VaxiJen server (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) was used for the prediction of protective antigens and subunit vaccines. According to the physicochemical properties of proteins, this server classifies antigens without referral to sequence alignment. The accuracy of the server based on the origin of the protein (bacterial, viral, and tumor protein datasets) varies between 70 and 89%^[22]. Antigenicity of the construct was rechecked by ANTIGENpro (<http://scratch.proteomics.ics.uci.edu/>),

QSPMTVLC SIDWFMVTVHPFMLNNDVCVHFHELHLGLGCPPNHVQPHAY
QFTYRVTECGIRAKAVSQDMVIYSTEIHYSKGTSPKFVIPVSCAAPQKSPW
LTKPCSMRVASKSRATAQKDEKCYEVFSLSQSSQRPNCDCPPCVFSEEHQ
VPCHQAGAQAQPLQPSHFLDISEDWSLHTDDMIGSMGSGGSGGSGGSGM
AQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINS AKDDAAGQAIAN
RFTANIKGLTQASRNANDGISIAQTTEGALNEINNQLQRVRELAVQSANST
NSQSDLDISIQAETQRLNEIDRVSGQTQFNGVKVLAQDNLTIQVGANDGE
TIDIDLKQINSQTLGLDTLNVQKQKVS DTAATVTGYADTTIALDNSTFKA
SATGLGGTDQKIDGDLKFDDTTGKYAKVTVTGGTGKDGYYEVSVDKTN
GEVTLAGGATSPLTGGLPATATEDVKNVQVANADLTEAKAALTAAGVTG
TASVVKMSYTDNNGKTIDGGLAVKVGDDYYSATQNKDGSISINTTKYTAD
DGTSKTALNKLGGADGKTEVVSIGGKTYAASKAEGHNFKAQPDLAEAAT
TTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRI
EDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQNVLSLLR

Fig. 1. Amino acid composition of PLAC1-fliC construct. Red shows PLAC1, black indicates a flexible linker, and green shows fliC.

which is based on pathogen independent, sequence-based, alignment-free analysis and uses antigenicity microarray data for predicting the protein antigenicity. SDAP (http://fermi.utmb.edu/SDAP/sdap_man.html) and AllergenFP (<http://www.ddgpharmfac.net/AllergenFP/>) databases were employed for allergenicity prediction of the fusion protein. SDAP is the structural database of allergenic proteins, while AllergenFP online bioinformatics tool is based on descriptor fingerprint^[23].

MHC-I and MHC-II binding epitope prediction

NetMHC 4.0 Server (<http://www.cbs.dtu.dk/services/NetMHC/>) and NetMHCII 2.3 Server (<http://www.cbs.dtu.dk/services/NetMHCII/>) were applied to predict MHC-I binding epitopes (based on an artificial neural networks method) and MHC-II binding epitopes, respectively^[24,25].

B cell and CTL epitopes prediction

B-cell epitopes, both continuous and discontinuous, were predicted using ElliPro server (<http://tools.iedb.org/ellipro/>)^[26]. CTLpred server (<http://crdd.osdd.net/raghava/ctlpred/>) was utilized for the prediction of CTL epitopes based on the direct method. This method uses data on T-cells epitopes templates instead of MHC-binding peptides. CTLpred method is based on techniques such as artificial neural network and support vector^[27].

RESULTS

Physicochemical properties

The fusion construct was designed (Fig. 1). The predicted molecular weight and isoelectric point for our fusion protein were 73642.54 Da and 5.17, respectively. Total numbers of negatively and positively charged amino acids of this protein were 73 and 55; thus, its final charge was -18, and it totally had a negative charge. Instability index for PLAC1-fliC protein was 32.87, which was considered as the stable protein; the instability index under 40 means stability. Aliphatic index and grand average of hydropathicity of our fusion protein were 76.85 and -0.380, respectively.

Secondary structure

Our findings showed that PLAC1-fliC had 29.31% alpha helix, 21.98% extended strand, and 48.71% random coil. Comparison between PLAC1, fliC, and PLAC1-fliC is shown in Table 1, and the secondary structure pattern of PLAC1-fliC is indicated in Figure 2.

Homology model building, refinement, and validation

The PLAC1-fliC protein model was built using Phyre2 based on Hidden Markov Model. Figure 3 depicts fusion protein predicted tertiary structure. This model was refined using GalaxyRefine server, and five

Table 1. Comparison of PLAC1, fliC, and PLAC1-fliC fusion secondary structures

Protein	Alpha helix (%)	Extended strand (%)	Random coil (%)
PLAC1	5.26	36.32	58.42
fliC	39.27	16.19	44.53
PLAC1-fliC	29.31	21.98	48.71

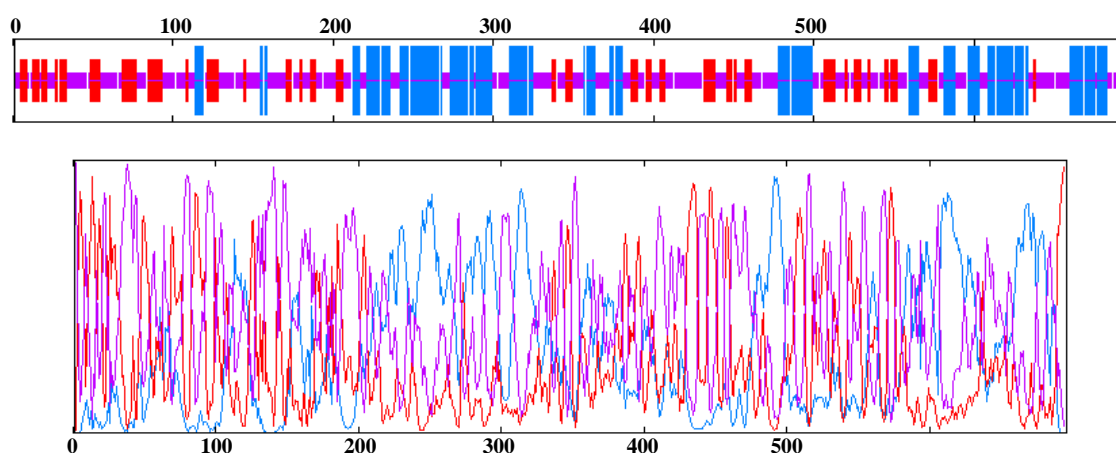


Fig. 2. Graphical results of secondary structures comparison. Helix, extended strand, and random coil are indicated by blue, purple and red, respectively.

refined models were built, and the best one was chosen. ProSA Z-scores for PLAC1-fliC 3D model after and before refinement were -8.38 and -9.21, respectively (Fig. 4). RAMPAGE results showed that the numbers of residues increased in the favored region after the model refinement. These findings were confirmed by PROCHECK results (Table 2).

Antigenicity and allergenicity

ANTIGENpro and VaxiJen results revealed that fliC, as an adjuvant, could increase the antigenicity of PLAC1 protein. Table 3 shows the antigenicity of PLAC1, fliC, linker, and PLAC1-fliC fusion protein. In accordance with AllergenFP server, our findings suggested that PLAC1, fliC, and PLAC1-fliC could not be an allergen for human body. Searching PLAC1, fliC, and PLAC1-fliC sequences in SDAP database showed that these sequences were not allergens, and, therefore, AllergenFP results were confirmed.

MHC-I and MHC-II-binding epitopes

Since 9-mer peptides display more tendency to HLA1, peptides with nine amino acids were chosen. The 9-mer peptides with strong binding preference to HLA-A0201 subtype with their affinity and rank are shown in Table 4. To predict MHC-II binding, 15-mer peptides binding to DRB1_0301, DRB1_0401, DRB1_0701, and DRB1_1501 were evaluated. Our finding exhibited that PLAC1-fliC epitopes were strong binders to DRB1_0301 and DRB1_0401 (Table 5).

B cell and CTL epitopes

Using ElliPro, B cell specific continuous (linear) and discontinuous (conformational) epitopes were defined. Accordingly, 16 continuous epitopes were identified,

and their length was between 6-110 amino acids (Table 6). In addition, 4 discontinuous epitopes were predicted with maximum 177 and minimum 15 amino acids in length (Table 7). Top 10 CTL peptide epitopes with higher scores predicted by combined approach, artificial neural network/support vector machine, are shown in Table 8.

DISCUSSION

According to the global cancer statistics in 2018, breast cancer is the most commonly diagnosed and the leading cause of cancer-related death among women^[1]. However, there are merely few therapeutic strategies with limited success against breast cancer^[28]. One new therapeutic strategy against cancer is immunotherapy. Cancer vaccine is one of the most effective immunotherapy treatments, but its design and production is often time-consuming and costly. The new generation of vaccines are based on recombinant

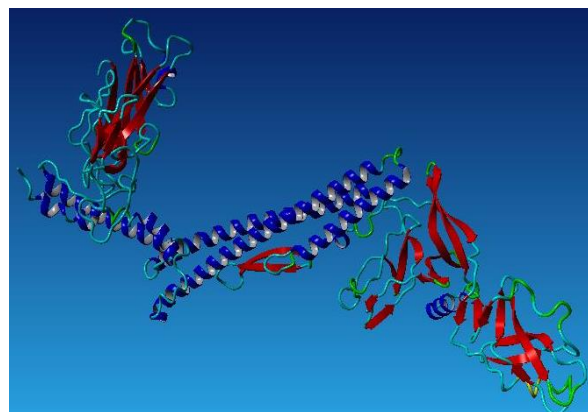


Fig. 3. The 3D structure of PLAC1-fliC defined by Phyre 2.

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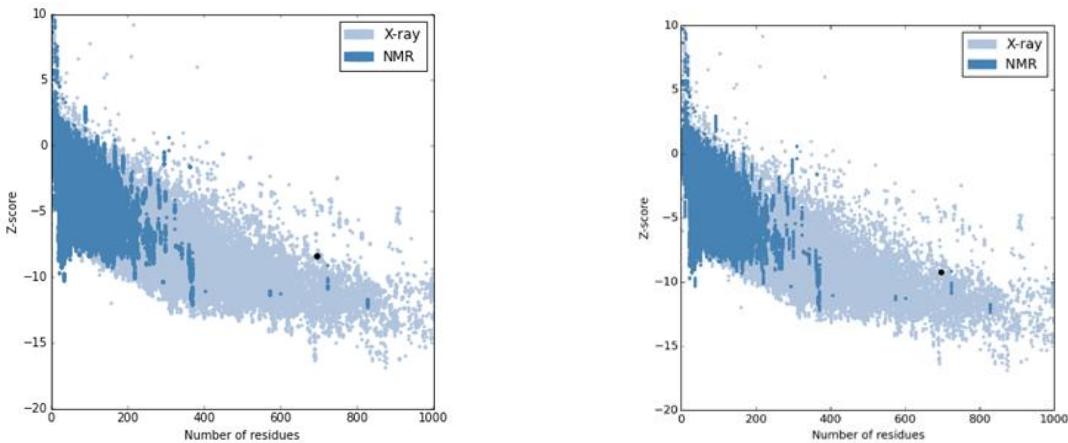


Fig. 4. The Z-score plot for the 3D model of PLAC1-fliC before (A) and after (B) refinement. Z-score is shown by a black spot.

protein technology according to their specific design, safety, purity, and feasibility of production^[29]. Thus, finding and screening the putative vaccine candidate is a prerequisite for its production. In a previous study, Kawada *et al.*^[30] vaccinated 13 patients using NY-ESO-1, a cancer-testis antigen. This antigen induces antibody production, as well as CD4 and CD8 T-cell responses. Furthermore, Shim *et al.*^[31] showed that cancer-associated peptides, as a cancer/testis antigen, can stimulate CTL. Thanks to the integration of biology and computer sciences, there are many available software and webserver that enable us to design and validate biological processes in a dry lab as *in silico*. Hence, we designed a candidate vaccine as a fusion protein, containing PLAC1, which is expressed highly in several types of tumor tissues, but not in normal ones, except placenta and testis^[32]. PLAC1 is a suitable candidate for cancer immunotherapy, since it is overexpressed in more than 80% of breast cancers samples, while overexpression of Her2/neu, the target of Herceptin monoclonal antibody, is utmost 25%^[8,33]. Second part of this fusion protein vaccine is fliC from *Salmonella enterica*, which as an adjuvant molecule

might be able to stimulate both humoral and cellular immune responses^[34]. Third part of this construct is a flexible linker, GSGGSGGSGGSG, which resides between the mentioned parts and contains repeated small or polar amino acids (glycine and serine). Such linkers provide favorable solubility and flexibility and join two domains of fusion protein without changing their structure and function^[35,36].

The construct structure was analyzed and validated using different bioinformatics tools and servers. Based on the results of different physicochemical parameters and structural analysis, PLAC1-fliC is a stable hydrophilic protein with a net negative charge. Its favorite degree of stability enables it to circulate easily and to be processed by antigen-presenting cells because proteins with a very loose conformation are susceptible to tertiary conformational epitope changes and might degrade easily. On the other hand, rigid structures might resist against antigen processing and epitope presentation^[37].

Secondary structures of polypeptides play an important role in their ultimate structure and function. Forecasting secondary structure from their amino acids

Table 2. Comparison of residues residence in Ramachandran plot before and after model refinement

Database	Before refinement	After refinement
RAMPAGE		
Number of residues in		
Favored region (%)	603 (86.9)	640 (92.2)
Allowed region (%)	54 (7.8)	33 (4.8)
Outlier region (%)	37 (5.3)	21 (3.0)
PROCHECK		
Residues in		
Most favored regions (%)	500 (81.2)	528 (85.7)
Additional allowed regions (%)	81 (13.1)	63 (10.2)
Generously allowed regions (%)	23 (3.7)	14 (2.3)
Disallowed regions (%)	12 (1.9)	11 (1.8)

Table 3. Antigenicity predictions of PLAC1, fliC, linker, and PLAC1-fliC.

Protein	ANTIGENpro	VaxiJen*
PLAC1	0.947422	0.6587
fliC	0.923670	0.8246
linker	0.453669	1.8025
PLAC1-fliC	0.955086	0.7320

* Threshold = 0.5

sequences is the prerequisite for proteins 3D structure prediction, as well as for prediction of protein stability. GOR V server was used to predict the secondary structure of PLAC1-fliC. It works according to Garnier-Osguthorpe-Robson method that uses combined mathematical tools, probability methods, and data have been extracted from empirical methods like NMR and crystallography^[38]. Table 1 reveals that the percentage of PLAC1-fliC random coil and alpha helix reduced and ioncreased, respectively. Therefore, its structure got more regular in comparison with PLAC1. Indeed, alpha helices are more stable than beta strands and random coils and more firm to conformational changes^[39]. PLAC1-fliC modeling, refinement and validation were accomplished via different servers. Based on RAMPAGE results, most residues were located in allowed and favorite regions, and even the scores got better after refinement. These results were validated by PROCHECK, as well (Table 2). The predicted model of PLAC1-fliC, when compared with the experimental structures, indicated that this model was valid and matched with experimentally determined structures. Appropriate ProSA Z-scores confirms the accuracy of this model (Fig. 4)^[20].

Since the presence of flagella protein domain, as an adjuvant in the construct, might be allergenic for human; it is necessary to evaluate its potential allergenicity. It is important that vaccines could not be an allergen for humans. Allergens provoke hosts immune system and might lead to undesirable allergenic reactions^[40]. The allergenicity prediction result showed that PLAC1-fliC was not allergen for humans; therefore, it can be administrated safely.

For stimulating immune responses against cancer cells and eventually eradicating them, their specific

antigen epitopes have to be presented to immune cells, including B and T lymphocytes. Intrinsic antigens like cancer epitopes are presented to CTL by MHC-I, and extrinsic antigen such as adjuvants are presented to T helper cells by MHC-II molecules. Commonly, MHC-I presents 8-11 amino acids of peptides, while MHC-II presents peptides with 12-25 amino acids in length; they interact with T-cell receptor and eventually lead to cellular immune response^[41]. The MHC genes are one of the most polymorphic human genes, and their frequency varies among populations, races, and ethnic groups. To predict the presenting potency and binding affinity of PLAC1-fliC in antigen-presenting cells, we selected the MHC-I and MHC-II alleles based on their global and regional relative haplotypes frequency according to scientific reports by investigating their frequency in allele frequency database^[42-46]. As Table 4 and 5 show, immunodominant 9-mer peptide of PLAC1-fliC strongly binds to MHC-I haplotype HLA-A0201, and 15-mer epitope of PLAC1-fliC binds to DRB1_0301 and DRB1_0401 MHC-II haplotypes. These results exhibit that PLAC1-fliC could be presented efficiently to immune cells and could evoke immunity against cancer cells. In addition, circulating antigens like protein-based vaccines could act as B-cell epitope and result in B-cell activation, antibody production, and humoral immunity stimulation. B-cell epitope is an antigenic determinant, recognized by antibodies or B-cell receptors. It can be a peptide, called the linear or continuous epitope, or it can have a 3D structure which is considered as a conformational or discontinuous epitope. B-cell epitopes of an antigen are antibody production stimulators^[26]. Based on Tables 6-8, PLAC1-fliC has both linear and conformational epitopes with strong affinity to B and T cells; therefore, it could potentially evoke humoral and cellular immunity.

In conclusion, our investigation regarding probable vaccine for breast cancer reveals that PLAC1-fliC contains suitable structure and stability that could effectively stimulate both cellular and humoral immunity and might be safe to administer. Since our study is a bioinformatics analysis, experimental studies are warranted for validity of our results.

Table 4. HLA1-binding peptides based on NetMHC 4.0

Peptide sequence	Position	HLA	Affinity (nM)	Rank (%)	Binding level
FMLNNDVCV	19	HLA-A0201	7.53	0.07	SB
FMVTVHPFM	12	HLA-A0201	10.81	0.12	SB
SIDWFMVTV	8	HLA-A0201	26.82	0.40	SB
VLCSIDWFM	5	HLA-A0201	35.00	0.50	SB

SB, strong binder

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Table 5. HLA2-binding peptides based on NetMHCII 2.3

Peptide sequence	Position	HLA	Affinity (nM)	Rank (%)	Binding level
DTTIALDNSTFKASA	1	DRB1_0301	15.5	0.50	SB*
ADTTIALDNSTFKAS	2	DRB1_0301	17.0	0.50	SB
TTIALDNSTFKASAT	3	DRB1_0301	17.3	0.60	SB
YADTTIALDNSTFKA	4	DRB1_0301	22.0	0.80	SB
HPFMLNNDVCVHFHE	5	DRB1_0301	22.7	0.80	SB
PFMLNNDVCVHFHEL	6	DRB1_0301	24.3	0.90	SB
VHPFMLNNDVCVHFH	7	DRB1_0301	27.2	1.10	SB
TIALDNSTFKASATG	8	DRB1_0301	33.6	1.40	SB
FMLNNDVCVHFHELH	9	DRB1_0301	40.2	1.70	SB
GYADTTIALDNSTFK	10	DRB1_0301	42.3	1.80	SB
QNRFNSAITNLGNTV	1	DRB1_0401	6.3	0.01	SB
VQNRFNSAITNLGNT	2	DRB1_0401	6.6	0.01	SB
NRFNSAITNLGNTVN	3	DRB1_0401	7.3	0.03	SB
AVQNRFNSAITNLGN	4	DRB1_0401	8.1	0.04	SB
RFNSAITNLGNTVNN	5	DRB1_0401	11.7	0.12	SB
GAVQNRFNSAITNLG	6	DRB1_0401	16.1	0.30	SB
DSDYATEVSNMSRAQ	7	DRB1_0401	17.7	0.40	SB
SDYATEVSNMSRAQI	8	DRB1_0401	19.0	0.40	SB
EDSDYATEVSNMSRA	9	DRB1_0401	19.7	0.50	SB
IEDSDYATEVSNMSR	10	DRB1_0401	27.9	0.90	SB
DTTIALDNSTFKASA	11	DRB1_0401	29.5	1.00	SB
DYATEVSNMSRAQIL	12	DRB1_0401	30.2	1.00	SB
TTIALDNSTFKASAT	13	DRB1_0401	33.7	1.20	SB
ADTTIALDNSTFKAS	14	DRB1_0401	33.9	1.20	SB
VHPFMLNNDVCVHFH	15	DRB1_0401	42.5	1.70	SB
GHNFKAQPDLAEEAT	16	DRB1_0401	44.9	1.80	SB
TVHPFMLNNDVCVHF	17	DRB1_0401	45.4	1.90	SB
RAQILQQAGTSVLAQ	1	DRB1_0101	8.3	1.90	SB

*SB strong binder

Table 6. Predicted B-cell linear epitopes of PLAC1-fliC

No.	Start	End	Peptide	Number of residues	Score
1	378	487	QQKYKVSDTAATVTGYADTTIALDNSTFKASATGLGG TDQKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYY EVSVDKTNGEVTLAGGATSPLTGGLPATATEDVKNVQVA	110	0.815
2	76	90	IHYSSKGTSPKFVIP	15	0.787
3	14	51	MVTVHPFMLNNDVCVHFHELHLGLGCPPNHVQPHAYQF	38	0.778
4	1	6	QSPMTV	6	0.74
5	237	261	GLRINSAKDDAAGQAIANRFTANIK	25	0.726
6	173	194	LDISEDWSLHTDDMIGSMGSGG	22	0.708
7	679	696	SVLAQANQVPQNVLSLLR	18	0.69
8	151	168	HTQVPCHQAGAQAQPLQ	18	0.662
9	513	523	SYTDNNGKTID	11	0.654
10	107	123	SMRVASKSRATAQKDEK	17	0.649
11	298	310	AVQSANSTNSQSD	13	0.637
12	196	222	GGSGGSGMAQVINTNSLSLLTQNNLNK	27	0.61
13	138	145	NCDCPPCV	8	0.593
14	603	615	ATTENPLQKIDA	13	0.588
15	647	658	NLTSARSRIEDS	12	0.585
16	347	359	TIQVGANDGETID	13	0.581

Table 7. Predicted B-cell discontinuous epitopes of PLAC1-fliC

No.	Residues	Number of residues	Score
1	_:A298, _:V299, _:Q300, _:S301, _:A302, _:N303, _:S304, _:T305, _:N306, _:S307, _:Q308, _:D310, _:V377, _:Q378, _:Q379, _:Y381, _:K382, _:V383, _:S384, _:D385, _:T386, _:A387, _:A388, _:T389, _:V390, _:T391, _:G392, _:Y393, _:A394, _:D395, _:T396, _:T397, _:I398, _:A399, _:L400, _:D401, _:N402, _:S403, _:T404, _:F405, _:K406, _:A407, _:S408, _:A409, _:T410, _:G411, _:L412, _:G413, _:G414, _:T415, _:D416, _:Q417, _:K418, _:I419, _:D420, _:G421, _:D422, _:L423, _:K424, _:F425, _:D426, _:D427, _:T428, _:T429, _:G430, _:K431, _:Y432, _:Y433, _:A434, _:K435, _:V436, _:T437, _:V438, _:T439, _:G440, _:G441, _:T442, _:G443, _:K444, _:D445, _:G446, _:Y447, _:Y448, _:E449, _:V450, _:S451, _:V452, _:D453, _:K454, _:T455, _:N456, _:G457, _:E458, _:V459, _:T460, _:L461, _:A462, _:G463, _:G464, _:A465, _:T466, _:S467, _:P468, _:L469, _:T470, _:G471, _:G472, _:L473, _:P474, _:A475, _:T476, _:A477, _:T478, _:E479, _:D480, _:V481, _:K482, _:N483, _:V484, _:Q485, _:V486, _:A487, _:S513, _:T515, _:D516, _:N517, _:N518, _:G519, _:K520, _:T521, _:I522, _:D523, _:D542	133	0.787
2	_:Q1, _:S2, _:P3, _:M4, _:T5, _:V6, _:M14, _:V15, _:T16, _:V17, _:H18, _:P19, _:F20, _:M21, _:L22, _:N23, _:N24, _:D25, _:V26, _:C27, _:V28, _:H29, _:F30, _:H31, _:E32, _:L33, _:H34, _:L35, _:G36, _:L37, _:G38, _:C39, _:P40, _:P41, _:N42, _:H43, _:V44, _:Q45, _:P46, _:H47, _:A48, _:Y49, _:Q50, _:F51, _:V55, _:S66, _:Q67, _:D68, _:M69, _:I76, _:H77, _:Y78, _:S79, _:S80, _:K81, _:G82, _:T83, _:P84, _:S85, _:K86, _:F87, _:V88, _:I89, _:P90, _:Q97, _:K98, _:S99, _:T103, _:C106, _:S107, _:M108, _:R109, _:V110, _:A111, _:S112, _:K113, _:S114, _:R115, _:A116, _:T117, _:A118, _:Q119, _:K120, _:D121, _:E122, _:N138, _:C139, _:D140, _:C141, _:P142, _:P143, _:C144, _:V145, _:H151, _:T152, _:Q153, _:V154, _:P155, _:C156, _:H157, _:Q158, _:A159, _:G160, _:A161, _:Q162, _:E163, _:A164, _:Q165, _:P166, _:L167, _:Q168, _:L173, _:D174, _:I175, _:S176, _:E177, _:D178, _:W179, _:S180, _:L181, _:H182, _:T183, _:D184, _:D185, _:M186, _:I187, _:G188, _:S189, _:M190, _:G191, _:S192, _:G193, _:G194, _:S195, _:G196, _:G197, _:S198, _:G199, _:G200, _:S201, _:G202, _:M203, _:A204, _:Q205, _:V206, _:I207, _:N208, _:T209, _:N210, _:S211, _:L212, _:S213, _:L214, _:L215, _:Q217, _:N218, _:N219, _:N221, _:K222, _:S679, _:V680, _:L681, _:A682, _:Q683, _:A684, _:N685, _:Q686, _:V687, _:P688, _:Q689, _:N690, _:V691, _:L692, _:S693, _:L694, _:L695, _:R696	177	0.687
3	_:G237, _:L238, _:R239, _:I240, _:N241, _:S242, _:A243, _:K244, _:D245, _:D246, _:A247, _:A248, _:G249, _:Q250, _:A251, _:I252, _:A253, _:N254, _:R255, _:F256, _:T257, _:A258, _:N259, _:K261, _:T347, _:Q349, _:V350, _:G351, _:A352, _:N353, _:D354, _:G355, _:E356, _:T357, _:D359, _:T644, _:N647, _:L648, _:S650, _:A651, _:R652, _:S653, _:R654, _:I655, _:E656, _:D657, _:S658, _:Y660	48	0.667
4	_:G371, _:L372, _:L375, _:A569, _:A602, _:A603, _:T604, _:T605, _:T606, _:E607, _:N608, _:P609, _:L610, _:Q611, _:K612	15	0.500

Table 8. Predicted CTL epitopes of PLAC1-fliC

Peptide rank	Start position	Sequence	Score (ANN/SVM)*
1	9	SIDWFMVTV	0.73/1.2411843
2	36	GLGCPPNHV	0.96/0.71105708
3	61	RAKAVSQDM	0.97/0.5147798
4	83	TPSKFVIPV	0.88/0.5965804
5	56	TECGIRAKA	0.85/0.5788711
6	87	FVIPVSCAA	0.94/0.37276101
7	70	VIYSTEIHY	0.82/0.4671223
8	22	LNNDVCVHF	0.57/0.59104808
9	119	QKDEKCYEV	0.95/0.14603929
10	6	VLCSIDWFM	0.46/0.53298459

*Cut-off score: 0.51 (ANN) and 0.36 (SVM); ANN, artificial neural network; SVM, support vector machine

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CONFLICT OF INTEREST. None declared.

REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians* 2018; **68**(6): 394-424.
- Zhou J, Zhong Y. Breast cancer immunotherapy. *Cellular and molecular immunology* 2004; **1**(4): 247-255.
- Song Q, Zhang CD, Wu XH. Therapeutic cancer vaccines: From initial findings to prospects. *Immunology letters* 2018; **196**: 11-21.
- Chang WL, Yang Q, Zhang H, Lin HY, Zhou Z, Lu X, Zhu C, Xue LQ, Wang H. Role of placenta-specific protein 1 in trophoblast invasion and migration. *Reproduction* 2014; **148**(4): 343-352.
- Kulkarni P, Shiraishi T, Rajagopalan K, Kim R, Mooney SM, Getzenberg RH. Cancer/testis antigens and urological malignancies. *Nature reviews urology* 2012; **9**(7): 386-396.
- Afsharpad M, Nowroozi MR, Ayati M, Saffari M, Nemati S, Mohebbi E, Nekooheh L, Zendehehdel K, Modarressi MH. ODF4, MAGEA3, and MAGEB4: Potential Biomarkers in Patients with Transitional Cell Carcinoma. *Iranian biomedical journal* 2018; **22**(3): 160-170.
- Li Y, Chu J, Li J, Feng W, Yang F, Wang Y, Zhang Y, Sun C, Yang M, Vasilatos SN, Huang Y, Fu Z, Yin Y. Cancer/testis antigen- Plac1 promotes invasion and metastasis of breast cancer through Furin/NICD/PTEN signaling pathway. *Molecular oncology* 2018; **12**(8): 1233-1248.
- Koslowski M, Sahin U, Mitnacht-Kraus R, Seitz G, Huber C, Türeci Ö. A placenta-specific gene ectopically activated in many human cancers is essentially involved in malignant cell processes. *Cancer research* 2007; **67**(19): 9528-9534.
- Yuan H, Chen V, Boisvert M, Isaacs C, Glazer RI. PLAC1 as a serum biomarker for breast cancer. *PLoS one* 2018; **13**(2): e0192106
- Hekmat S, Sadat SM, Aslani MM, Mahdavi M, Bolhassani A, Asgar Halvae F, Ghahari SMM, Aghasadeghi MR, Siadat SD. Truncated core/NS3 fusion protein of HCV adjuvanted with outer membrane vesicles of *Neisseria meningitidis* serogroup B: Potent inducer of the murine immune system. *Iranian biomedical journal* 2019; **23**(4): 235-245.
- Cui B, Liu X, Fang Y, Zhou P, Zhang Y, Wang Y. Flagellin as a vaccine adjuvant. *Expert review of vaccines* 2018; **17**(4): 335-349.
- Atapour A, Mostafavi-Pour Z, Mokarram P, Mohammadi S. Cloning, expression and purification of a novel fusion protein composed of flagellin and NS5B of hepatitis C virus in *Escherichia coli* host. *Shiraz E-medical journal* 2018; **20**(1): e79898.
- Khani MH, Bagheri M, Zahmatkesh A, Bidhendi SM. Immunostimulatory effects of truncated and full-length flagellin recombinant proteins. *Microbial pathogenesis* 2019; **127**: 190-197.
- Yoon SI, Kurnasov O, Natarajan V, Hong M, Gudkov AV, Osterman AL, Wilson IA. Structural basis of TLR5-flagellin recognition and signaling. *Science* 2012; **335**(6070): 859-864.
- Song L, Xiong D, Kang X, Yang Y, Wang J, Guo Y, Xu H, Chen S, Peng D, Pan Z, Jiao X. An avian influenza A (H7N9) virus vaccine candidate based on the fusion protein of hemagglutinin globular head and Salmonella typhimurium flagellin. *BMC biotechnology* 2015; **15**(1): 79.
- Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. Protein Identification and Analysis Tools on the ExPASy Server. The proteomics protocols handbook. Germany: Springer; 2005.
- Sen TZ, Jernigan RL, Garnier J, Kloczkowski A. GOR V server for protein secondary structure prediction. *Bioinformatics* 2005; **21**(11): 2787-2788.
- Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. *Nature protocols* 2015; **10**(6): 845-858.
- Lee GR, Heo L, Seok C. Effective protein model structure refinement by loop modeling and overall relaxation. *Proteins* 2016; **84**(Suppl 1): 293-301.
- Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic acids research* 2007; **35**(Web Server issue): W407-W410.
- Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of applied crystallography* 1993; **26**(2): 283-291.
- Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC bioinformatics* 2007; **8**: 4.
- Dimitrov I, Naneva L, Doytchinova I, Bangov I. AllergenFP: allergenicity prediction by descriptor fingerprints. *Bioinformatics* 2014; **30**(6): 846-851.
- Andreatta M, Nielsen M. Gapped sequence alignment using artificial neural networks: application to the MHC class I system. *Bioinformatics* 2015; **32**(4): 511-517.
- Jensen KK, Andreatta M, Marcantili P, Buus S, Greenbaum JA, Yan Z, Sette A, Peters B, Nielsen M. Improved methods for predicting peptide binding affinity to MHC class II molecules. *Immunology* 2018;

- 154(3): 394-406.
26. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, Sette A, Peters B. ElliPro: a new structure-based tool for the prediction of antibody epitopes. *BMC bioinformatics* 2008; **9**(1): 514.
 27. Bhasin M, Raghava G. Prediction of CTL epitopes using QM, SVM and ANN techniques. *Vaccine* 2004; **22**(23-24): 3195-3204.
 28. Hutchinson L. Breast cancer: challenges, controversies, breakthroughs. *Nature reviews clinical oncology* 7(2): 669-670.
 29. Vergati M, Intrivici C, Huen NY, Schlom J, Tsang KY. Strategies for cancer vaccine development. *BioMed research international* 2010; **2010** Article ID 596432.
 30. Kawada J, Wada H, Isobe M, Gnjjatic S, Nishikawa H, Jungbluth AA, Okazaki N, Uenaka A, Nakamura Y, Fujiwara S, Mizuno N, Saika T, Ritter E, Yamasaki M, Miyata H, Ritter G, Murphy R, Venhaus R, Pan L, Old LJ, Doki Y, Nakayama E. Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination. *International journal of cancer* 2012; **130**(3): 584-592.
 31. Shim E, Shim H, Bae J, Lee H, Jeoung D. CAGE displays oncogenic potential and induces cytolytic T lymphocyte activity. *Biotechnology letters* 2006; **28**(7): 515-522.
 32. Silva WA Jr., Gnjjatic S, Ritter E, Chua R, Cohen T, Hsu M, Jungbluth AA, Altorki NK, Chen YT, Old LJ, Simpson AJG, Caballero OL. PLAC1, a trophoblast-specific cell surface protein, is expressed in a range of human tumors and elicits spontaneous antibody responses. *Cancer immunity* 2007; **7**: 18.
 33. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; **244**(4905): 707-712.
 34. Delavari S, Sohrabi M, Ardestani MS, Faezi S, Tebianian M, Taghizadeh M, Shajiei A, Hosseini SY, Moghaddampour M, Mahdavi M. Pseudomonas aeruginosa flagellin as an adjuvant: superiority of a conjugated form of flagellin versus a mixture with a human immunodeficiency virus type 1 vaccine candidate in the induction of immune responses. *Journal of medical microbiology* 2015; **64**(11): 1361-1368.
 35. Chen X, Zaro JL, Shen WC. Fusion protein linkers: property, design and functionality. *Advanced drug delivery reviews* 2013; **65**(10): 1357-1369.
 36. Hachikubo Y, Ito K, Schiefelbein J, Manstein DJ, Yamamoto K. Enzymatic activity and motility of recombinant *Arabidopsis* myosin XI, MYA1. *Plant and cell physiology* 2007; **48**(6): 886-891.
 37. Scheiblhofer S, Laimer J, Machado Y, Weiss R, Thalhamer J. Influence of protein fold stability on immunogenicity and its implications for vaccine design. *Expert review of vaccines* 2017; **16**(5): 479-489.
 38. Kouza M, Faraggi E, Kolinski A, Kloczkowski A. The GOR method of protein secondary structure prediction and its application as a protein aggregation prediction tool. *Methods in molecular biology* 2017; **1484**: 7-24.
 39. Abrusán G, Marsh JA. Alpha helices are more robust to mutations than beta strands. *PLoS computational biology* 2016; **12**(12): e1005242.
 40. Kelso JM, Greenhawt MJ, Li JT, Nicklas RA, Bernstein DI, Blessing-Moore J, Cox L, Khan D, Lang DM, Oppenheimer J, Portnoy JM, Randolph C, Schuller DE, Spector SL, Tilles SA, Wallace D. Adverse reactions to vaccines. *Annals of allergy, asthma and immunology* 2009; **103**(1): 25-43.
 41. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews immunology* 2011; **11**(12): 823-836.
 42. Amirzargar A, Mytilineos J, Farjadian S, Doroudchi M, Scherer S, Opelz G, Ghaderi A. Human leukocyte antigen class II allele frequencies and haplotype association in Iranian normal population. *Human immunology* 2001; **62**(11): 1234-1238.
 43. González-Galarza FF, Takeshita LY, Santos EJ, Kempson F, Maia MHT, da Silva AL, Teles e Silva AL, Ghattaoraya GS, Alfievic A, Jones AR, Middleton D. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic acids research* 2014; **43**(Database issue): D784-D788.
 44. Esmaeili A, Rabe SZT, Mahmoudi M, Rastin M. Frequencies of HLA-A, B and DRB1 alleles in a large normal population living in the city of Mashhad, Northeastern Iran. *Iranian journal of basic medical sciences* 2017; **20**(8): 940-943.
 45. Varzi AM, Shahsavari F, Tarrahi MJ. Distribution of HLA-DRB1 and HLA-DQB1 alleles in Lak population of Iran. *Human immunology* 2016; **77**(7): 580-583.
 46. Moatter T, Aban M, Tabassum S, Shaikh U, Pervez S. Molecular analysis of human leukocyte antigen class I and class II allele frequencies and haplotype distribution in Pakistani population. *Indian journal of human genetics* 2010; **16**(3): 149-153.