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Original Article

In Silico Design and Evaluation of PRAME+FliC ΔD2D3 as a New Breast Cancer Vaccine Candidate

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What's Known

- Standard therapies used to treat breast cancer are only effective in approximately half of the patients.
- Among various immunotherapy methods, vaccination has gained much attention in treating breast cancer.

What's New

- Preferentially expressed antigen in melanoma (PRAME) combined with FliCΔD2D3 is a stable and soluble chimeric protein.
- PRAME+FliCΔD2D3 stimulated humoral and cellular immunity against breast cancer.

Abstract

Background: The most prevalent cancer in women over the world is breast cancer. Immunotherapy is a promising method to effectively treat cancer patients. Among various immunotherapy methods, tumor antigens stimulate the immune system to eradicate cancer cells. Preferentially expressed antigen in melanoma (PRAME) is mainly overexpressed in breast cancer cells, and has no expression in normal tissues. FliCΔD2D3, as truncated flagellin (FliC), is an effective toll-like receptor 5 (TLR5) agonist with lower inflammatory responses. The objective of the present study was to utilize bioinformatics methods to design a chimeric protein against breast cancer.

Methods: The physicochemical properties, solubility, and secondary structures of PRAME+FliCΔD2D3 were predicted using the tools ProtParam, Protein-sol, and GOR IV, respectively. The 3D structure of the chimeric protein was built using I-TASSER and refined with GalaxyRefine, RAMPAGE, and PROCHECK. ANTIGENpro and VaxiJen were used to evaluate protein antigenicity, and allergenicity was checked using AlgPred and Allergen FP. Major histocompatibility complex (MHC) and cytotoxic T-lymphocytes (CTL) binding peptides were predicted using HLApred and CTLpred. Finally, B-cell continuous and discontinuous epitopes were predicted using ABCpred and ElliPro, respectively.

Results: The stability and solubility of PRAME+FliCΔD2D3 were analyzed, and its secondary and tertiary structures were predicted. The results showed that the derived peptides could bind to MHCs and CTLs. The designed chimeric protein possessed both linear and conformational epitopes with a high binding affinity to B-cell epitopes.

Conclusion: PRAME+FliCΔD2D3 is a stable and soluble chimeric protein that can stimulate humoral and cellular immunity. The obtained results can be utilized for the development of an experimental vaccine against breast cancer.

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Keywords ● PRAME antigen ● Vaccines ● Breast neoplasms ● Computer simulation

Introduction

Breast cancer is considered as the most common women's cancer worldwide, and is the second leading cause of death after lung cancer.[1] The World Health Organization (WHO) has estimated 2.1 million new cases of breast cancer each year, and has reported

627,000 related deaths in 2018.[2, 3] Such a high number of deaths has been attributed to tumor progression and metastasis to other organs. Breast cancer is a heterogeneous disease, which requires targeted therapy consistent with the tumor's characteristics.[4] Standard therapies used to treat breast cancer (e.g., surgery, radiation therapy, anti-estrogen therapy, and chemotherapy; individually or in combination) are only effective in approximately half of the patients. It has been shown that these therapies pose adverse side-effects, since they do not target specific tumor cells.[5-7] Immunotherapy is a targeted approach and a promising method to treat cancer patients. Among various immunotherapy methods, vaccination has gained much attention in treating breast cancer. Recently, it has been shown that the use of antigen-specific vaccines are effective in eradicating breast tumor cells.[8, 9]

Preferentially expressed antigen in melanoma (PRAME) is a human tumor antigen found in melanomas. PRAME is also known as melanoma antigen, which is preferentially expressed in tumors (MAPE), OPA-interacting protein 4 (OIP4), and cancer-testis antigen 130 (CT130). The human PRAME gene is located on chromosome 22q11.22 and encodes a protein of 509-amino acids.[10] PRAME induces cell proliferation in melanoma cells by repressing retinoic acid (RA) signaling.[11] It belongs to the cancer-testis antigen (CTA) family based on its chromosomal position, expression, and immunogenicity. Typically, PRAME has no expression in normal tissues. However, low level of expression has been observed in some organs such as testis, ovaries, adrenal glands, and endometrium.[12] Aberrant expression of PRAME has been demonstrated in various cancers such as neuroblastoma,[13] ovarian adenocarcinomas,[14] head and neck cancer,[15] acute and chronic leukemia,[10] and breast cancer.[16] Previous studies have reported the overexpression of PRAME in breast cancer, making it a suitable prognostic and predictive biomarker. It is viewed as a potential candidate for immunotherapeutic strategies.[4, 16]

The goal of vaccination is to provide a strong immune response for long-term protection against antigens. To achieve this goal, a vaccine requires an adjuvant. Adjuvants are used to enhance the immune response against the vaccine and are classified in various groups. Toll-like receptors (TLR) ligands are known as a popular group of adjuvants that improve the immunogenicity of vaccines.[17, 18] Bacterial flagellins (also termed FliC) is considered as an effective adjuvant candidate. *Salmonella typhimurium* FliC, a principal structural protein of flagella, is identified as a TLR5 ligand. FliC stimulates several innate immune cells to release specific cytokines and chemokines, which in turn stimulate an adaptive immune response. FliC is a 494-amino acid protein consisting of two separate domains, namely D0/D1 and D2/D3. The domain D0/D1 (with 170 and 90 amino acids) plays a role in TLR5 agonist activity, and the domain D2/D3 (with 170 and 400 amino acids) is essential for flagellin antigenicity.[19-21] Various studies have shown the high antigenicity and inflammatory effects of FliC. As a result, truncated flagellin (FliCΔD2D3) has been designed by removing the hypervariable D2/D3 domain. In comparison to FliC, FliCΔD2D3 exhibits remarkably lower inflammatory responses, TLR5 agonist potency, more efficient immunoreactivity, and least allergenicity.[22, 23]

The design and development of a new vaccine are still time-consuming and requires extensive experimental investigations. Among various approaches, bioinformatics methods have been utilized to design more effective vaccines.[24] The present study aimed to employ bioinformatics methods to design a chimeric protein as a novel recombinant construct against breast cancer, composed of PRAME as the antigenic fragment and FliCΔD2D3 as the adjuvant part.

Materials and Methods

The present study was conducted in 2019 at the School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran. A complete amino acid sequence of PRAME was obtained from the UniProt Knowledgebase (http://www.uniprot.org, UniProt ID: P78395). FliCΔD2D3 (D0/D1+D1/D0) sequence was in accordance with a previous study.[22] The construct included PRAME at N-terminus connected to FliCΔD2D3.

Physicochemical Properties and Solubility

Different physicochemical characteristics, such as theoretical isoelectric point (pI), the total number of positive and negative residues, molecular weight (MW), instability index, aliphatic index (AI), and grand average hydropathy (GRAVY) of the chimeric protein were computed using the ProtParam web server (https://web.expasy.org/protparam). Protein solubility was evaluated using the Protein-sol web server (http://scratch.proteomics.ics.uci.edu/).

Secondary Structure Prediction

The secondary structure of PRAME, FliCΔD2D3 (D0/D1+D1/D0), and PRAME+

FliCΔD2D3 (D0/D1+D1/D0) proteins was predicted using the GOR IV web server with an accuracy of 73.5% (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html).

3D Model Building, Refinement, and Docking

The I-TASSER web server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to build a 3D model of the chimeric protein. The server is a combined platform for computerized protein structure and function prediction. The optimal 3D model from I-TASSER was used for refinement using the GalaxyRefine web server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). Validation of the 3D model of the chimeric structure was performed using the PROCHECK and RAMPAGE web servers. The PROCHECK (http://servicesn.mbi.ucla.edu/PROCHECK/) was used to check the stereochemical quality of the protein structure, and its results were verified using the RAMPAGE web server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). The interaction between the 3D model of the chimeric protein and TLR5 was predicted using the ZDOCK web server with >70% accuracy.

Antigenicity and Allergenicity Evaluation

The ANTIGENpro and VaxiJen web servers were used to evaluate the antigenicity of PRAME, FliCΔD2D3, and the chimeric protein. ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) is a sequence-based alignment-free and pathogen-independent predictor, which uses protein antigenicity microarray data for predicting protein antigenicity. VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used for the anticipation of conserved regions of antigens, and candidate vaccines according to physiochemical properties of proteins, neglecting alignment of sequences. The allergenicity of the chimeric protein was analyzed using the AlgPred and Allergen FP v.1.0 web servers. Allergen FP v.1.0 (http://ddg-pharmfac.net/Allergen FP) is a descriptor-based fingerprint bioinformatics tool. AlgPred (http://crdd.osdd.net/raghava/algpred/) predicts allergens based on similarity to known epitopes.

Prediction of MHC Binding Peptides

Major histocompatibility complex (MHC) class I and class II binding peptides were identified using the HLApred web server (http://crdd.osdd.net/raghava/hlapred/). The server predicts HLA binder peptides that can be potential vaccine candidates.

Prediction of B-Cell Epitopes

The ABCpred web server (http://crdd.osdd.net/raghava/abcpred/) was used to predict linear (continuous) epitopes with 65.93% accuracy. This server has been established through a machine-based technique, which considered a recurrent neural network via fixed-length patterns. ElliPro (http://tools.iedb.org/ellipro/) is an online bioinformatics web server that has been used for the prediction of conformational (discontinuous) antigens, based on the 3D structure of antigenic proteins. This structure-based server uses three algorithms, namely approximation of a protein surface patch by an ellipsoid, computation of the residue protrusion index (PI), and cluster neighboring residues based on their PI values.

Results

Physicochemical Analysis of PRAME+FliCΔD2D3 Protein

ProtParam analysis of PRAME+FliCΔD2D3 protein showed that the molecular weight and theoretical isoelectric point (pI) of the chimeric protein were approximately 87 kDa and 5.89 units, respectively. We found 79 negatively charged aspartate and glutamate amino acids, as well as 70 positively charged arginine and lysine in the protein sequence (i.e., the net charge of the protein was -9). Based on the computed instability index of 42.44, the chimeric protein was considered an unstable protein. The

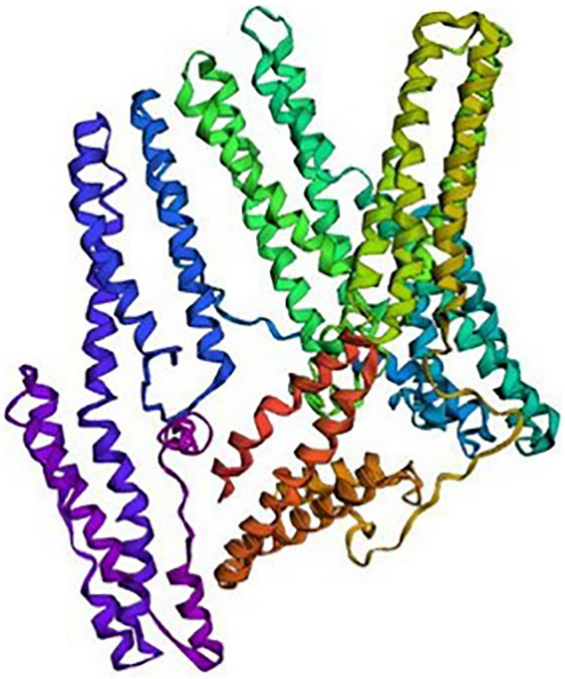

Figure 1: Tertiary structure of PRAME-FliCΔD2D3 is illustrated. The structure was modeled and built using I-TASSER homology-based modeling.

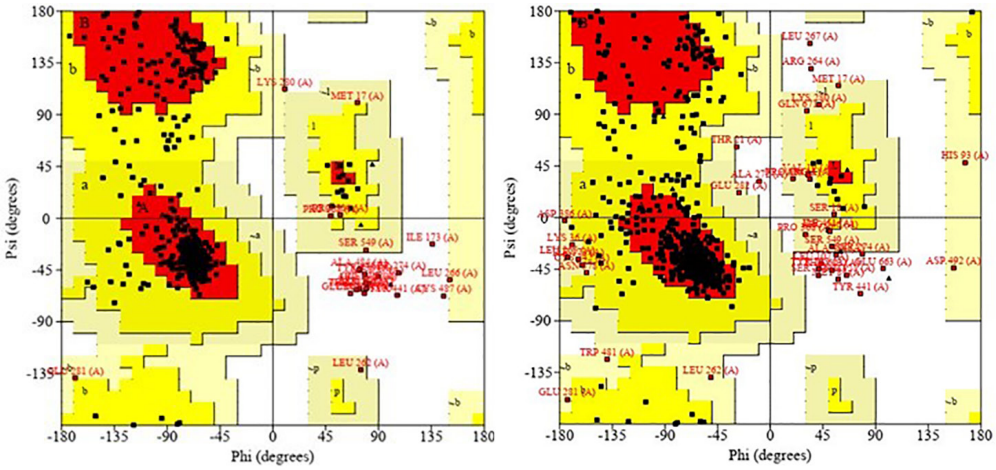

Figure 2: Ramachandran plot for the 3D structure before (left) and after (right) refinement using the PROCHECK web server is illustrated.

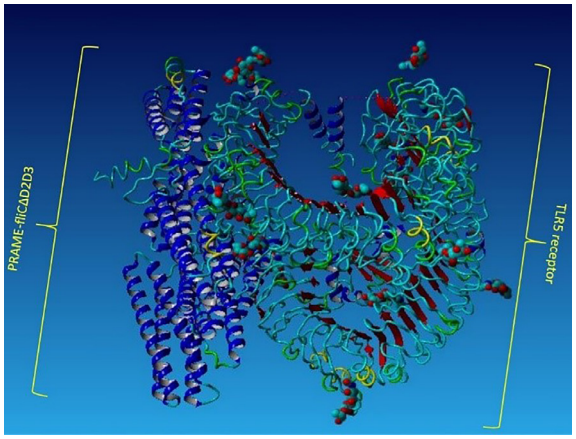

Figure 3: Docking of PRAME-FliCΔD2D3 and toll-like receptor (TLR5) shows suitable interaction between them.

AI and GRAVY indices were 101.63 and -0.107, respectively. The solubility of the protein was determined using the SOLpro web server. The result showed that this protein could be soluble with a probability of 0.585946 (cut-off: 0.5).

Secondary and Tertiary Structure

The results of GOR IV for the prediction of the secondary structure of the chimeric protein indicated that the PRAME consisted of 46.95% alpha-helix, 36.94% random coil, and 16.11% extended strand. Whereas the chimeric protein consisted of 50% alpha-helix, 37.34% random coil, and 12.66% extended strand. Therefore, the protein was mainly composed of regular structures. The 3D models of PRAME+FliCΔD2D3 protein were built using the I-TASSER web server. A model with the most positive c-score was chosen (data not shown). The putative tertiary structure is shown in figure 1.

3D Structure Refinement, Validation, and Docking

An optimal I-TASSER model was used as an input to the GalaxyRefine web server. This server suggested five refined protein models (data not shown) of which the model with the highest Rama favored score was chosen to carry out more analysis. Based on the Ramachandran plot, the results of PROCHECK and RAMPAGE web servers showed that residues in the favored and most favored regions were increased after model refinement (figure 2). The results of PROCHECK showed that residues in most favored regions before and after 3D model refinement were 80.6% and 88.5%, respectively. In addition, the results of RAMPAGE showed that residues in the favored region before and after 3D model refinement were 83.0% and 94.4%, respectively. PRAME+FliCΔD2D3 tertiary refined model and the TLR5 interaction showed that the chimeric vaccine could bind to TLR5 receptor (figure 3).

Antigenicity and Allergenicity

The results of ANTIGENpro showed that the antigenicity of the chimeric protein was increased compared to that of the PRAME alone (from 0.161 to 0.485, threshold: 0.4). In addition, using the VaxiJen web server, the predicted antigenicity of PRAME and the chimeric protein was 0.527 and 0.611 (threshold: 0.5), respectively. This implied that FliCΔD2D3 might increase the antigenicity of PRAME. The AlgPred and Allergen FP v.1.0 web servers predicted the non-allergenic potential of the chimeric protein.

MHC-I and MHC-II Binding Peptides

The HLApred web server predicted possible MHC-I binding peptides in the PRAME+FliCΔD2D3 chimeric protein. The position, sequence, and score of the 10 best-ranked peptides with the potential to bind to MHC-I are listed in table 1. Moreover, the predicted binding peptides, which had an affinity

Table 1: Major histocompatibility complex I binding peptides of preferentially expressed antigen in melanoma

Allele	Rank	Position	Sequence	Score	Prediction
HLA-A*0201 (threshold: 7.470)	1	371	ALLERASAT	12.900	Binder
	2	194	YLIEKVKRK	12.770	Binder
	3	190	ELFSYLIEK	12.390	Binder
	4	28	ELAGQSLLK	12.290	Binder
	5	237	DLEVTCTWK	12.230	Binder
	6	429	HLIGLSNLT	11.030	Binder
	7	118	KLQVLDLRK	10.780	Binder
	8	401	SLSHCSQLT	9.940	Binder
	9	469	ELLCELGRP	8.970	Binder
	10	68	TLKAMVQAW	8.710	Binder

Table 2: Major histocompatibility complex II binding peptides of PRAME-FliCΔD2D3

Allele	Rank	Position	Sequence	Score	Prediction
HLA-DRB1*0301 (threshold: 2.960)	1	223	IKMILKMVQ	5.200	Binder
	2	230	VQLDSIEDL	4.960	Binder
	3	383	LVFDECGIT	4.800	Binder
	4	301	LYVDSLFFL	4.360	Binder
HLA-DRB1*0401 (threshold: 1.480)	1	194	YLIEKVKRK	4.300	Binder
	2	738	YATEVSNMS	3.800	Binder
	3	321	MNPLETLSI	3.200	Binder
	4	430	LIGLSNLTH	3.180	Binder
HLA-DRB1*0701 (threshold: 4.100)	1	513	VINTNSLSL	8.200	Binder
	2	7	WGSIQSRYI	6.500	Binder
	3	669	LKQINSQTL	6.300	Binder
	4	238	LEVTCTWKL	5.720	Binder
HLA-DRB1*1501 (3% threshold=3.250)	1	259	MINLRRLLL	5.500	Binder
	2	262	LRRLLLSHI	4.600	Binder
	3	513	VINTNSLSL	4.300	Binder
	4	321	MNPLETLSI	4.160	Binder

PRAME: Preferentially expressed antigen in melanoma

to the DRB1_1101, DRB1_0301, and DRB1_1001 alleles of MHC-II are listed in table 2.

B-cells Epitopes

The ABCpred and ElliPro web servers predicted B-cell continuous (linear) and discontinuous (conformational) epitopes, respectively. Residue, position, and score of the predicted epitopes are listed in tables 3 and 4.

Discussion

In the present study, a chimeric protein was designed as a breast cancer vaccine candidate. PRAME is a suitable candidate for immunotherapy, since its expression increases in breast cancer cells, and it is a suitable prognostic and predictive biomarker.[4, 16] As an adjuvant FliCΔD2D3, a truncated FliC, exhibits less inflammatory properties and more efficient TLR5 potency than FliC. Therefore, we used FliCΔD2D3, as the adjuvant molecule capable of stimulating both the innate and adaptive immune responses.[22] This chimeric protein was then analyzed and evaluated using various bioinformatics web servers. Based on the physicochemical parameters and structural features of PRAME+FliCΔD2D3, the results showed an instability index of 42.44, indicating that the protein to be unstable (values above 40.0 are considered unstable). However, previous studies reported that value in the range of 40 to 45 (as in our case) could be considered stable.[25] Based on the calculated net charge (-9), our chimeric protein could be soluble. Note that the protein net charge is a significant physical property, which directly affects its solubility, aggregation, and crystallization. Since the body recognizes hydrophobic particles as foreign substances, and the reticulo-endothelial system removes them from the bloodstream, proteins with a net negative charge can mask the hydrophobicity and prevent the removal of the particles.[26] The net charge of proteins can also impact thermal stability, and the free energy interplay between proteins and ligands.[27] Depending on the desired level of stability and charge, PRAME+FliCΔD2D3 could be soluble, easily circulate in the bloodstream, be processed by antigen-presenting cells (APCs),

Table 3: B-cell continuous epitopes of Preferentially expressed antigen in melanoma (threshold: 0.5)

Rank	Sequence	Start position	Score
1	HCGDRTFYDPEPILCP	489	0.92
2	WGSIQSRYISMSVWTS	7	0.90
3	FPEPEAAQPMTKKRKV	146	0.87
4	PPLFMAAFDGRHSQTL	54	0.86
4	RPRRWKLQVLDLRKNS	113	0.86
5	DSIEDLEVTCTWKLPT	233	0.83
5	MQDIKMILKMVQLDSI	220	0.83
5	MSVWTSPRRLVELAGQ	17	0.83
5	FWTVWSGNRASLYSFP	132	0.83
6	DGRHSQTLKAMVQAWP	62	0.81
6	NCRLSEGDVMHLSQSP	331	0.81
7	LRHVMNPLETLSITNC	317	0.80
8	EVTCTWKLPTLAKFSP	239	0.79
9	ASSYISPEKEEQYIAQ	272	0.78
9	RASLYSFPEPEAAQPM	140	0.78
10	DECGITDDQLLALLPS	386	0.77
10	LKMVQLDSIEDLEVTC	227	0.77

Table 4: B-cell discontinuous epitopes of Preferentially expressed antigen in melanoma

No.	Residues	Number of residues	Score
1	A:H129, A:Q130, A:F132, A:W133, A:T134, A:V135, A:W136, A:S137, A:G138, A:N139, A:R140, A:A141, A:S142, A:L143, A:Y144, A:S145, A:F146, A:P147, A:E148, A:P149, A:E150, A:A151, A:A152, A:Q153, A:P154, A:M155, A:T156, A:K157, A:K158, A:R159, A:K160, A:V161, A:D162, A:G163, A:L206, A:R207, A:L208, A:C209, A:C210, A:K211, A:K212, A:L213, A:K214, A:I215, A:F216, A:A217, A:M218, A:P219, A:M220, A:Q221, A:D222, A:I223, A:K224, A:M225, A:L227, A:V230, A:A376, A:S377, A:A378, A:T379, A:L380, A:Q381, A:D382, A:L383, A:V384, A:F385, A:E387, A:C388, A:G389, A:I390, A:T391, A:D392, A:D393, A:Q394, A:L395, A:L396, A:V439, A:Y441, A:P442, A:V443, A:P444, A:L445, A:E446, A:S447, A:Y448, A:E449, A:D450, A:I451, A:H452, A:G453, A:T454, A:L455, A:H456, A:L457, A:E458, A:R459	96	0.731
2	A:M1, A:E2, A:R3, A:R4, A:R5, A:L6, A:W7, A:G8, A:S9, A:I10, A:Q11, A:S12, A:R13	13	0.603

and would not be easily removed from the body. The computed AI was 101.63. It is indicated that AI is related to amount of aliphatic side chains in a protein (A, V, I, and L), and is a positive factor for increased thermostability of a protein. Lower thermostability of PRAME+FliCΔD2D3 indicated a more flexible structure. The GRAVY index of PRAME+FliCΔD2D3 was as low as -0.107, indicating better interaction with water.[28]

The secondary structure of polypeptides plays a central role in their eventual formation and function. The GOR IV web server was used to predict the secondary structure of PRAME+FliCΔD2D3. The algorithm of this web server is based on the GOR (Garnier-Osguthorpe-Robson) method, which utilizes a combination of mathematical probability and experimental data (e.g., data from nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography).[29] The results from the GOR IV webserver showed that the addition of FliCΔD2D3, as an adjuvant, did not alter the secondary structure of the antigen. The percentage of alpha helices and random coils increased in PRAME+FliCΔD2D3, and its construction became more regular than that of PRAME alone (table 1). Among protein secondary structures, alpha helices are reported to be more stable and more resistant to conformational variations.[30] Various web servers were used to build, refine, and validate a 3D model of PRAME+FliCΔD2D3. Based on the RAMPAGE web server results, most residues were distributed in the favored and allowed regions, and the residues increased after refinement. These results were confirmed using the PROCHECK web server. The refined 3D model of protein docking of the chimeric vaccine showed that it could bind to TLR5 and stimulate APCs' augmentation and T-helper cells (Th) responses.

Since bacterial flagellin was used as an adjuvant in our chimeric vaccine, it was important to evaluate its potential allergenicity and ensure that it does not induce allergic responses in the human body. Allergenic substances may adversely affect the host's immune system and result in undesired allergic diseases. The results of the allergenicity assessment confirmed that

PRAME+FliCΔD2D3 did not induce an allergic response.

To elicit an immune response against tumor cells, immune cells such as B- and T-lymphocytes must be presented to antigen epitopes. MHC-I and MHC-II binding peptides can stimulate cytotoxic T-lymphocytes (CTL) and Th, respectively. The MHC human genes are among the most polymorphic genes. MHC-I and MHC-II alleles were used to determine the potential presentation and binding affinity of PRAME+FliCΔD2D3 by APCs. These alleles were selected because of their global and local comparative haplotypes frequency and their incidence in allele frequency database.[31-33] The position, sequence, and score of the 10 best-ranked peptides with MHC-I binding affinity were identified. Peptides that had the best binding affinity to DRB1-1101, DRB1-0301, and DRB1-1001 alleles of MHC-II were also identified. These confirmed that PRAME+FliCΔD2D3 could be effectively presented to T-cells and stimulate immunity against cancer cells.

Circulating soluble antigens such as vaccines could be composed of B-cells epitopes, which stimulate the generation of specific antibodies and activate humoral immunity. B-cell epitopes (antigenic determinants) are recognized by B-cell receptors or secreted antibodies. In terms of spatial structure, B-cell epitopes are categorized as continuous (linear) and discontinuous (conformational) epitopes. For the latter, amino acid residues are in close interaction because of the 3D structure. In fact, B-cell epitopes are the instigators of antibody generation.[34] As the results of the presented study indicated, the PRAME+FliCΔD2D3 chimeric protein possessed both linear and conformational epitopes with a strong affinity to B-cells, indicating that it can stimulate humoral immunity.

The main limitation of the present study was that we only performed *in silico* analysis. It is recommended that future studies experimentally validate our bioinformatics data.

Conclusion

Given its structure, PRAME+FliCΔD2D3 is a suitable candidate for a new breast cancer vaccine and could effectively stimulate both cellular and humoral immunity. It is recommended to validate this vaccine candidate through *in vitro* and *in vivo* studies.

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Conflict of Interests: None declared.

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