RESEARCH ARTICLE

Editorial Process: Submission:09/29/2024 Acceptance:01/19/2025

Screening of Oncogenic Proteins and Development of a Multiepitope Peptide Vaccine Targeting *AKT1* and *PARP1* for Breast Cancer by Integrating Reverse Vaccinology and Immune-Informatics Approaches

Sakshi Gupta[1], Drishti Desai[1], Manasi Patel[2], Charmi Jyotishi[2], Ahmad Mahmoud Saleh[3]*, Reeshu Gupta[1,2]*

Abstract

Background: Breast cancer remains a significant global health challenge, requiring innovative therapeutic strategies. In silico methods, which leverage computational tools, offer a promising pathway for vaccine development. These methods facilitate antigen identification, epitope prediction, immune response modelling, and vaccine optimization, accelerating the design process. **Methods:** This study employed a reverse vaccinology approach combined with various bioinformatic tools to design a multi-epitope peptide vaccine. **Results:** Using reverse vaccinology, *AKT1* and *PARP1* were identified as potential vaccine candidates, as their expression levels were significantly higher in breast cancer samples compared to healthy controls. The vaccine was designed by integrating immune cell epitopes with a TLR4 agonist as an adjuvant. It demonstrated high antigenicity, no allergenicity, and no toxicity. Validation of its 3D structure using the Ramachandran plot confirmed optimal conformation and stereochemical properties. Molecular docking and simulation studies showed the vaccine was stable and compact when interacting with TLR4. Moreover, the subunit vaccine effectively eliminated the antigen and triggered a strong IgG/IgM immune response lasting approximately one year (350 days). **Conclusion:** These findings suggest that the designed vaccine holds promise as a therapeutic option for breast cancer. However, further in vitro and in vivo studies are necessary to validate its efficacy before advancing to clinical trials.

Keywords: Breast cancer- Immunoinformatics- Reverse vaccinology- TLR4

Asian Pac J Cancer Prev, **26 (1)**, 327-338

Introduction

Breast cancer is the most frequently diagnosed cancer among women. According to GLOBOCAN 2022, it ranks as the second most common cancer globally, with 2,296,840 new cases and 666,103 deaths reported. The highest incidence rates were observed in Asia (42.9%), followed by Europe (24.3%) and North America (13.3%). Mortality rates were also highest in Asia (47.3%), with Europe (21.7%) and Africa (13.7%) following. Additionally, the 5-year survival rates are notably low for metastatic breast cancer (29%) and triple-negative breast cancer (TNBC) (12%), regardless of subtype [1]. Therefore, more effective and targeted treatments are required for BC.

Breast cancer treatment is usually performed via surgery, in addition to radiotherapy and chemotherapy. However, they are not effective in all patients due of their various molecular subtypes. Therefore, immunotherapies are currently gaining attention for the treatment of the disease. For example, trastuzumab (Herceptin) [2] and pertuzumab (Perjeta) [3] are antibodies that target HER2 dimerization. Another immunotherapy strategy involves vaccination, where a tumor-specific immune response is activated by targeting tumor-associated antigens. For example, E75 (HER2/neu 369–377: KIFGSLAFL) is a vaccine that can kill HER2 overexpressing tumor cells by activating T cells [4]. Briefly, antigen-containing genes stimulate B cells, helper T cells (HTLs), and cytotoxic T lymphocytes (CTLs) to elicit a specific immune response, contributing to targeted therapy. However, developing therapeutic vaccines for breast cancer faces significant challenges, including (1) the complexity of the immune system and (2) its inherent heterogeneity [5].

Traditional methods for developing breast cancer vaccines involve identifying tumor antigens, conducting

*[1]Parul Institute of Applied Sciences, Parul University, Vadodara, India. [2]Centre of Research for Development, Parul University, Vadodara, India. [3]Nursing College, Prince Sattam bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia. *For Correspondence: reeshu.gupta25198@paruluniversity.ac.in, alraminy@gmail.com*

preclinical studies, formulating vaccines, undergoing clinical trials, and securing regulatory approval. However, these approaches are often time-consuming, labour-intensive, expensive, and do not guarantee the desired efficacy. Reverse vaccinology has emerged as a possible solution to these limitations. Unlike conventional methods, reverse vaccinology leverages genomic data to design vaccines and has been successfully applied to develop multi-epitope subunit vaccines for various cancers. Peptide-based vaccines are gaining prominence due to their stability and effective delivery strategies, including protection during gastrointestinal transit. Multi-epitope vaccines offer distinct advantages, such as enhanced processing with MHC molecules, the ability to induce both humoral and cell-mediated immune responses, and broader HLA coverage compared to single-epitope vaccines [6].

In this study, we utilized a reverse vaccinology approach, employing various bioinformatic tools to design an in-silico multi-epitope vaccine candidate for breast cancer. These tools helped assess key factors such as transmembrane domains, allergenicity, antigenicity, toxicity, and the physiochemical properties of the antigens. *AKT1* and *PARP1* were identified as promising vaccine candidates. The final vaccine formulation included multiple immune epitopes and a TLR4 agonist as an adjuvant to activate both humoral and cell-mediated immunity. Molecular dynamics (MD) simulations and immune simulations further indicated strong immunogenic potential for the constructed vaccine.

Materials and Methods

Acquisition of the data

An extensive literature search was performed using various websites such as PubMed, MEDLINE, Embase, and clinicaltrials.gov. The following studies were excluded from the literature search: 1) no full-text availability; 2) duplication of genes; 3) no match between abstract and title; 4) no focus on cancers, vaccines, or drugs; 5) no statistically significant data; and 6) all the clinical trials that were terminated, suspended, or withdrawn. These criteria led to the identification of 36 relevant genes after screening 354 relevant clinical trials and 251 research papers to identify potential vaccine candidates against breast cancer. The reverse vaccinology approach was employed to identify potential vaccine candidates and develop the vaccine (Figure 1). Genes were selected based on their relevance to immunotherapeutic responses and tumor-suppressive properties.

Identification of surface proteins

The surface localizations of all 36 proteins were predicted using the VaxElan server as described previously by our group [7].

Trans-membrane (TM) analysis

Two tools, HMMTOP and TMHMM, have been used to screen transmembrane proteins as described previously by our group [7].

Instability index analysis

Protein stability was determined using the ProtParam tool. Proteins with value of instability index < 40 were selected.

Antigenicity prediction

Antigenicity was predicted using the VaxiJen v2.0 server as described previously [7]. Proteins with a value higher than the default threshold value of 0.4 were considered antigenic.

Non-allergenicity analysis

To identify the proteins with potential allergenic effects, the AllerTOP v. 2.0 was used to identify proteins with allergenic properties. Using the BLASTp program, only proteins with ≤ 35% identity scores were considered non-allergens [8, 9].

Evaluation of filtered protein

The RStudio's peptides' package and ProtParam tool was used to assess the physiological attributes of the vaccine candidate as described previously by our group [7].

Ethical approval

Five breast cancer patients referred by Parul Sevashram Hospital were enrolled in this study in 2023. Five healthy volunteers were included as the control group. The study was approved by the Ethics Committee of Parul University (PUIECHR/ PIMSR/00/081734/5308). All methods were performed according to the relevant guidelines and regulations provided by the Ethics Committee of Parul University.

Real time polymerase chain reaction

RNA extraction from five breast cancer patients was performed using TRIzol (Qiagen, Cat # 79306), as described previously [10]. cDNA was synthesized from

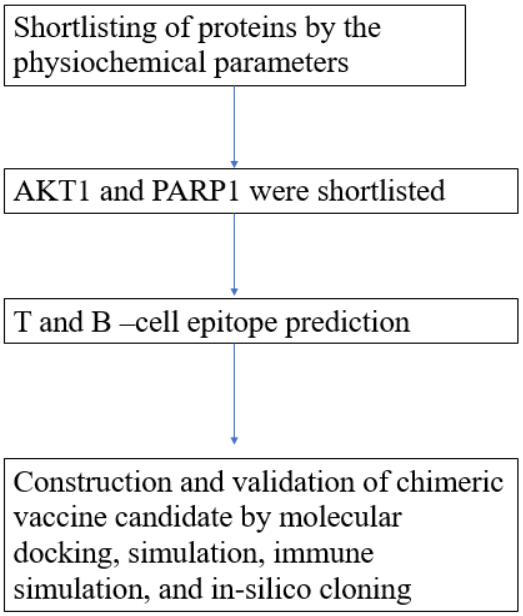

Figure 1. Flow Chart to Develop Potential Vaccine Candidates Using Reverse Vaccinology Approach

100 ng of RNA using a G-Biosciences cDNA Synthesis Kit. The resulting cDNA was used for qRT-PCR (Rotor-Gene Q, Qiagen). qRT-PCR was performed in triplicate with 2X SYBR Green qPCR Master Mix from G-Biosciences under the following conditions: 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The relative expression levels of the target gene mRNAs were calculated by the comparative CT method (relative expression = $2^{-\Delta\Delta CT}$) using β-actin as an internal control. Primer sequences were: human *AKT1* forward CCTCTCCATGCAGGAGTTAAGA, human *AKT1* reverse GGTCTCGGGTCCTTGATTTTCT; human *PARP1* forward TGGCCTCCATTGATGCTACC, human *PARP1* reverse GGACGACTTTGGGACGAACT; human beta actin forward GGACTTCGAGCAAGAGATGG, and human beta actin reverse AGCACTGTGTTGGCGTACAG.

Protein expression analysis

The subcellular localization and expression of *AKT1* and *PARP1* genes in CRC, OC, and oral cancer were further verified at the protein levels by analysing Human Protein Atlas database of immunohistochemistry (IHC) (https://www.proteinatlas.org/) using HPAanalyze R package [11].

Prediction of B-cell epitopes

B-cell epitopes were predicted using default values on the ABCPred server for the shortlisted proteins (*AKT1* and *PARP1*). Briefly, epitopes with 15 amino acid residues were predicted using a server [12].

T-cell Epitope prediction

The Immune Epitope Database (IEDB) was used to identify cytotoxic T-lymphocytes (CTL) epitopes using two methods 1) the stabilised matrix method (SMM) and 2) NetMHCPan 4.1 EL (epitope prediction) ((http://tools.iedb.org/mhci/). The same IEDB server and similar methods of SMM and NetMHCPan 4.1 EL were used to predict epitopes of helper T lymphocytes (HTL) using the default set of reference HLA alleles (http://tools.iedb.org/mhcii/). These tools predict the binding affinity of epitopes to MHC based on IC50 and percentile rank, as described elsewhere [13, 14].

Antigenicity, allergenicity, toxicity and immunogenicity of the immune epitopes

Antigenicity and allergenicity of the B and T cell epitopes were determined using servers described previously by our group [7]. ToxinPred predicts toxicity. Immunogenicity of the epitopes were predicted using the IFNepitope and IL-10Pred server on the basis of the production of IFN-γ and IL-10 cytokines [15].

Creation of the multi-epitope vaccine candidate

The multi-epitope vaccine was constructed by linking B and T cell epitopes with adjuvants using four linkers (EAAAY, AAY, GPGPG, and KK), as described previously [7]. A TLR4 agonist (50S ribosomal protein L7/L12; accession no: P9WHE3.1) was chosen as an adjuvant for the vaccine construct.

3D structure refinement and validation

The I-TASSER server was used to predict the 3-D model of the vaccine construct using LOMETS technology, which predicts the tertiary structure based on multiple relevant threads. The 3D structure of the vaccine was refined using GalaxyRefine and finally the structure was analysed using a Procheck Ramachandran plot to determine its stereochemical characteristics and optimal conformations [16]. Antigenicity, allergenicity, and toxicity of the refined vaccine were determined as described above.

Molecular docking of subunit vaccine with immune receptor

Molecular docking between the toll-like receptor (TLR4-PDB ID:4G8A) and the vaccine construct was performed using the ClusPro server [17]. Both proteins and ligands were prepared using the UCSF chimera by assigning charges, adding hydrogen, and removing water molecules [18]. Using the HPEPDOCK server, the binding affinities for both vaccine-TLR4 and peptides-protein complexes were evaluated [19].

Molecular dynamics simulation

MD simulations were performed by applying an OPLS force field using the GROMACS version 2022.5 [20]. The simulation was performed for 150 ns, and the root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), and solvent-accessible surface area (SASA) parameters were determined.

Characterisation of immune responses of the construct

The C-immSim immune server with default settings was used to predict the immune responses of the vaccine construct. Briefly, three antigenic injections at regular intervals of 4 weeks were administered to observe the immune response, as described previously [21].

Codon adaptation of vaccine construct

The Java Codon Adaptation Tool (JCat server) was used to optimize codons in Homo-sapiens, as described previously [22]. Finally, Snap Gene v4.2 software (https://snapgene.com/) was used to insert the modified sequences into the pET28a (+) expression vector using the restriction enzymes NmeAIII and BseY1. Snap Gene v4.2 (https://snapgene.com/).

Results

In this study, we used reverse vaccinology (RV) and bioinformatics tools to identify potential vaccine candidates against Breast Cancer.

To identify suitable vaccine candidate using Reverse vaccinology pipeline

Of the RV pipeline out of 36 proteins, *AKT1* and *PARP1* (accession ID: NCT03742102) with protein RAC-alpha serine/threonine-protein kinase and Poly [ADP-ribose] polymerase1 were shortlisted as potential vaccine candidates against breast cancer. The shortlisted proteins do not have any transmembrane domain and are

Table 1. B-cell Epitopes Present on Surfaces of *AKT1 & PARP1*

Protein	Antigenic B-cell epitopes	Antigenic score	Allergenicity	Toxicity	IFN Server	IL10 inducer
AKT1	KEKATGRYYAMKILKK	0.5	N	N	Positive/0.489	Positive/0.423
PARP1	REISYLKKLKVKKQDR	1.1941	N	N	Positive/0.890	Positive/ 1.087

non-allergic, antigenic, or non-toxic in nature (Table 1). Hydrophilic epitopes are surface antigens that can produce an immunogenic response by interacting with immune cells. These results suggest that both *AKT1* and *PARP1* are potential vaccine candidates for breast cancer (Supplementary Table 1). Additionally, negative GRAVY values and low instability indices suggest their hydrophilic and stable characteristics (Supplementary Table 2).

Real time qPCR analysis of AKT1 and PARP1

To determine the significance of *AKT1* and *PARP1* in liquid biopsy samples of breast cancer, we assessed the expression levels of these genes in serum samples. As shown in Figure 2a-2b, the expression of *AKT1* (control: 0.74 ± 0.34; patients: 1.10 ± 0.42), *PARP1* (control: 1.12 ± 0.59; patients: 1.59 ± 0.33), was markedly higher in breast cancer samples when compared to normal controls.

AKT1 and PARP1 protein expression analysis

The results of the HPAanalyze demonstrated that *AKT1* was localized to the nucleoplasm and microtubules, while PARP1 was localized to the nucleoplasm and nucleoli (Figure 3a). The analysis also revealed that approximately 0.90 proportions of patients showed high *PARP1* expression compared to *AKT1* (0.35) (Figure 3b).

Prediction of B-cell epitopes

B cell epitopes activate humoral immunity against various diseases and infections. The ABCPred server predicted 48 and 110 B cell epitopes in *AKT1* and *PARP1*, respectively. Of these 158 epitopes, only one epitope in each gene was shortlisted based on their high antigenicity, non-allergenicity, and non-toxicity. Additionally, these epitopes induced IFN-γ and IL-10 production (Table 1). While IFN-γ activates CTL, IL-10 is an inflammatory cytokine with pleiotropic functions, including T-cell activation and tumor rejection [23].

T-cell epitopes

CD8+ T-cell epitopes

Cytotoxic T-cell epitopes (CD8+ epitopes) processed and presented along with MHC I. Three CD8+ epitopes in each of *AKT1* and *PARP1* proteins were shortlisted on the basis of their high antigenicity, no allergenicity, and non-toxicity profile (Table 2a).

Furthermore, the PEP-FOLD server was used to predict the 3D structure of CD8+ epitopes or peptides, and the binding scores of these peptides to TLR4 were calculated using the HPEPDOCK server. The binding scores of KTWRPRYF, LLKKDPKQRLG, and NQDHEKLFEL epitopes of *AKT1* were - 229.029, - 168.306, - 189.364, respectively, while DELKKVCS, KLYRVEYAK, and KVCSTNDLK epitopes of *PARP1* had binding score of - 197.227, - 152.668, and -118.015, respectively.

CD4+ T-cell epitopes

In total, five CD4+ epitopes (*AKT1*:1 and PAPR1:4) in both proteins were shortlisted. These epitopes exhibited high antigen scores, no allergenicity, and no toxicity (Table 2b). The binding affinities of EEMEVSLAKPKHRVT, FREISYLKKLKVKKQ, KEFREISYLKKLKVK, EFREISYLKKLKVKK, and REISYLKKLKVKKQD epitopes to TLR4 were -184.203, -196.098, - 216.115, - 188.227, and -172.908, respectively.

Evaluation of vaccine constructs

The antigenicity score of the vaccine construct either with or without adjuvant was higher than the threshold value of 0.5 (0.76 with adjuvant, and 0.8365 without adjuvant). Additionally, the vaccine construct was hydrophilic (GRAVY value = -0.497), stable (instability index = 13.02), non-allergenic, and non-toxic. It had a 55.9% (232 amino acids) helix, 9.39% (39 amino acids) beta-sheets, and 34.69% (144 amino acids) turns, as predicted by the PSIPRED tool.

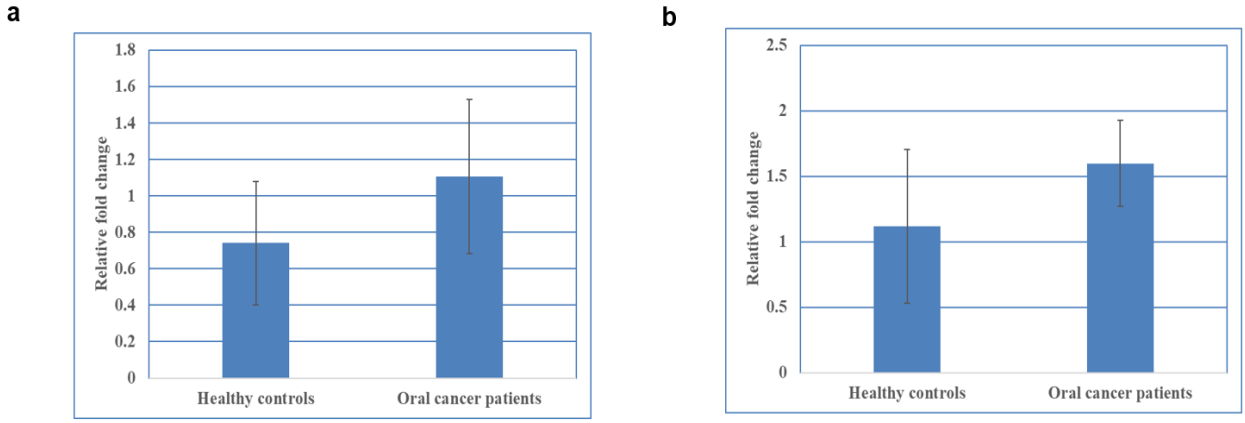

Figure 2. qPCR Analysis of a) *AKT1* and b) *PARP1* genes in breast cancer samples and normal controls

Table 2A. List of Top Scoring MHC Class I Binding Cytotoxic T-cell Epitopes in *AKT1* and *PARP1*

Target protein	MHC Class I binding epitopes	ALLELE (NetMHCpan 4.1)	ALLELE (SMM)	*AS	*PS	*PCS
AKT1	KTWRPRYFLLK	HLA-A*23:01	HLA-A*32:01			
		HLA-B*58:01	HLA-C*12:03	1.13	1.84	1.31
		HLA-A*30:01	HLA-A*11:01			
		HLA-B*08:01	HLA-A*30:01			
	LLKKDPKQRLG	HLA-A*31:01	HLA-C*12:03			
		HLA-B*08:01		0.64	0.21	0.81
		HLA-A*02:03				
		HLA-B*08:01				
	NQDHEKLFEL	HLA-B*08:01	HLA-C*05:01			
		HLA-B*40:01	HLA-C*12:03	1.2	1.81	1.38
		HLA-A*02:06				
		HLA-B*08:01				
PARP1	KLYRVEYAK	HLA-A*30:01	HLA-A*11:01			
		HLA-A*31:01	HLA-C*12:03	1.36	1.22	0.9
		HLA-A*32:01	HLA-C*14:02			
		HLA-A*68:01				
	KVCSTNDLK	HLA-A*11:01	HLA-A*11:01			
		HLA-A*03:01	HLA-C*12:03	1.8	1	0.74
		HLA-A*30:01				
		HLA-A*68:01				

AS, Antigenic score; PS, Proteosome score; PCS, Processing score

Prediction, refinement and stereochemical attributes of the tertiary structure

The 3D model of the vaccine construct was predicted using the I-TASSER server with the 10 best threading templates: 1rquA, 7apjA, 2ftcF, 5h7cA, 8ugcA, 7f4Ua, 1dd3A0, 6d03E, 8f7nA, and 1dd3A from the PDB library. The C-scores of these models ranged from -1.69 to -4.16. The model with the highest c-score was selected for refinement. The original model was refined using GalaxyRefine server. The accuracy of the model was defined using the Global Distance Test High Accuracy (GDT-HA), MolProbity (highlights steric clashes), and poor rotamers. GalaxyRefine generated five models after refinement, of which Model 1 was selected owing to the lower scores of GDT-HA, MolProbity, and poor rotamers in comparison to the original one (Refined: GDT-HA: 0.9958, MolProbity: 1.956, poor rotamers: 0.6; Original: GDT-HA: 0.9958, MolProbity: 1.956, poor rotamers: 0.6).

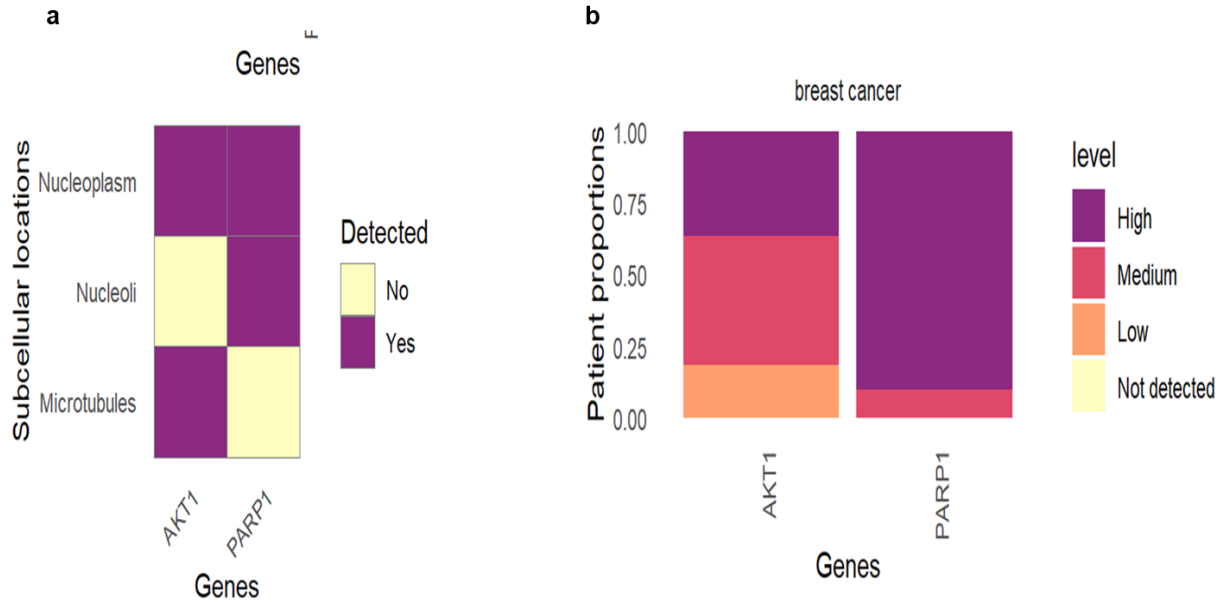

Figure 3. a) Subcellular localization of CDK2 and ID1 b) Patient proportions of AKT1 and PARP1 in breast cancer. The data was analyzed using HPAanalyze R package in R studio.

Table 2b. List of Top Scoring MHC Class II Binding Cytotoxic T-cell Epitopes in *AKT1* and *PARP1*

Target protein	MHC Class I binding epitopes	ALLELE (NetMHCpan 4.1)	ALLELE (SMM)	*AS	Allergen	Toxic
AKT1	EEMEVSLAKPKHRVT	HLA-DRB5*01:01	HLA-DRB1*03:01			
		HLA-B*58:01	HLA-DRB1*07:01	0.62	No	No
		HLA-A*30:01	HLA-DRB1*15:01			
		HLA-B*08:01	HLA-DRB3*01:01			
PARP1	FREISYLKKLKVKKQ	HLA-DRB1*11:01	HLA-DRB5*01:01			
		HLA-DRB5*01:01	HLA-DRB4*01:01	1.06	No	No
			HLA-DRB1*15:01			
			HLA-DRB1*03:01			
	KEFREISYLKKLKVK	HLA-DRB1*11:01	HLA-DRB5*01:01			
		HLA-DRB5*01:01	HLA-DRB1*15:01	0.88	No	No
			HLA-DRB1*07:01			
			HLA-DRB1*03:01			
	EFREISYLKKLKVKK	HLA-DRB1*11:01	HLA-DRB5*01:01			
		HLA-DRB5*01:01	HLA-DRB4*01:01			
			HLA-DRB3*02:02	1.1	No	No
			HLA-DRB1*15:01			
	REISYLKKLKVKKQD	HLA-DRB1*11:01	HLA-DRB1*15:01			
		HLA-DRB5*01:01	HLA-DRB1*03:01			
			HLA-DRB5*01:01	1.14	No	No
			HLA-DRB4*01:01			

AS, Antigenic score

In the case of the original model, 88.9%, 6.8%, 1.3%, and 3% of residues in the Ramachandran plot were observed in the favored, allowed, generously allowed, and disallowed regions, respectively. However, the Ramachandran plot predicted that 98.7% of the residues were in the favored and allowed regions, while 0.3% and 1.1% of the residues were in the generously allowed and disallowed regions, respectively, in the refined model.

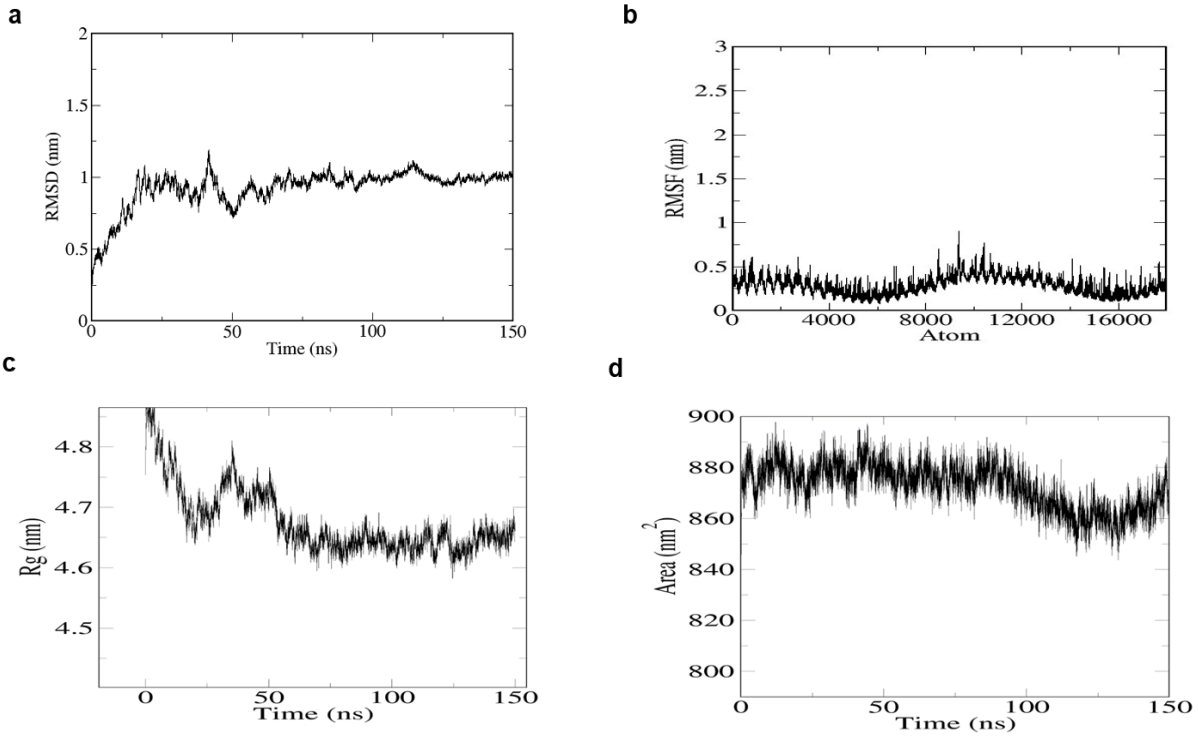

Figure 4. Molecular Dynamics Simulation Study of TLR4-Vaccine Construct. a) Root mean square deviation b) Root mean square fluctuations c) Radius of gyration d) Solvent accessible surface area for the time duration of 150 ns.

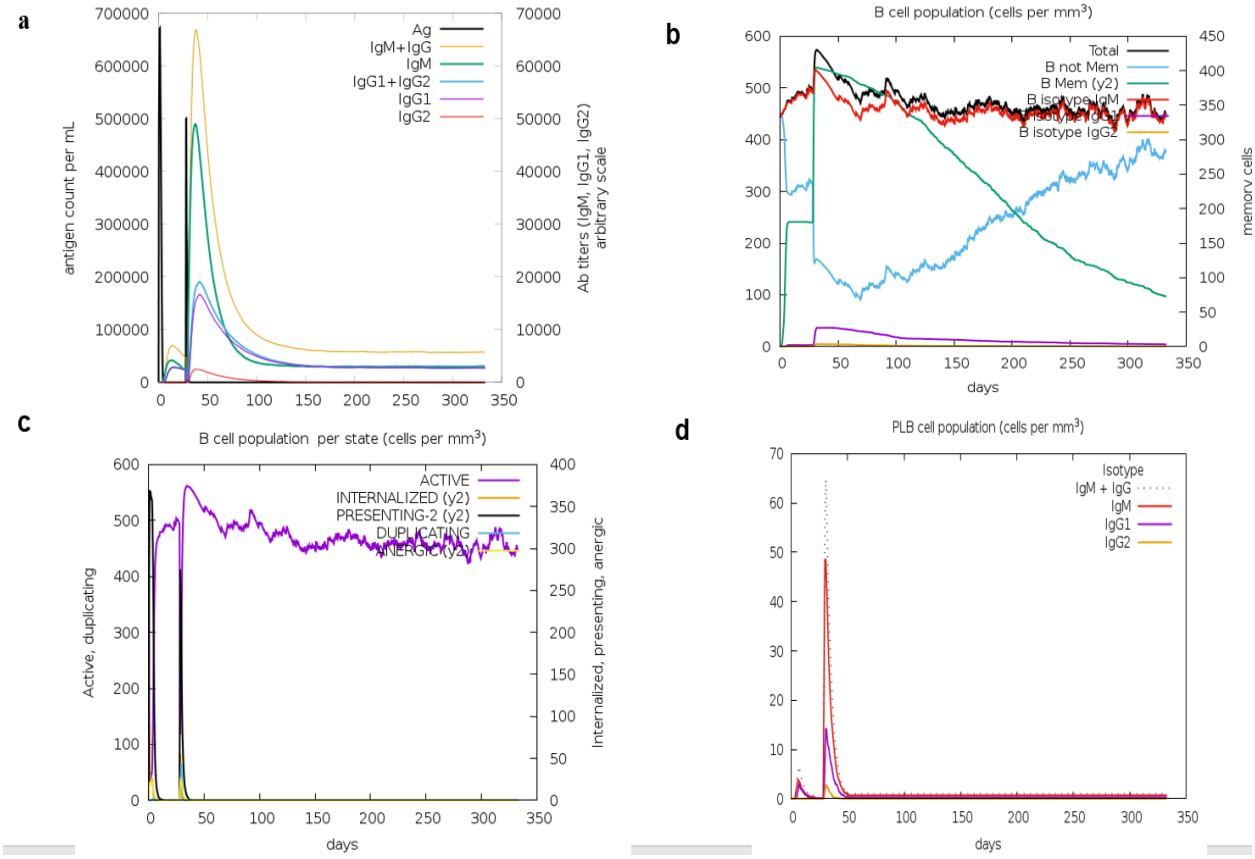

Figure 5. a) Figure 3 a) Immunoglobulin subclasses in response to vaccine injection b) B cell population (cells per mm3) c) B cell population per stated (cells per mm^3) d) PLB cell population (cells per mm3)

The ERRAT server was used to analyze the statistics of non-bonded interactions. The ERRAT score was set at 81.27 for the original model and 100 for the refined model. Generally, an ERRAT score greater than 50 represents a good-quality model; therefore, a score of 100 validates our modelled structure. ProSA-web showed a Z-score of

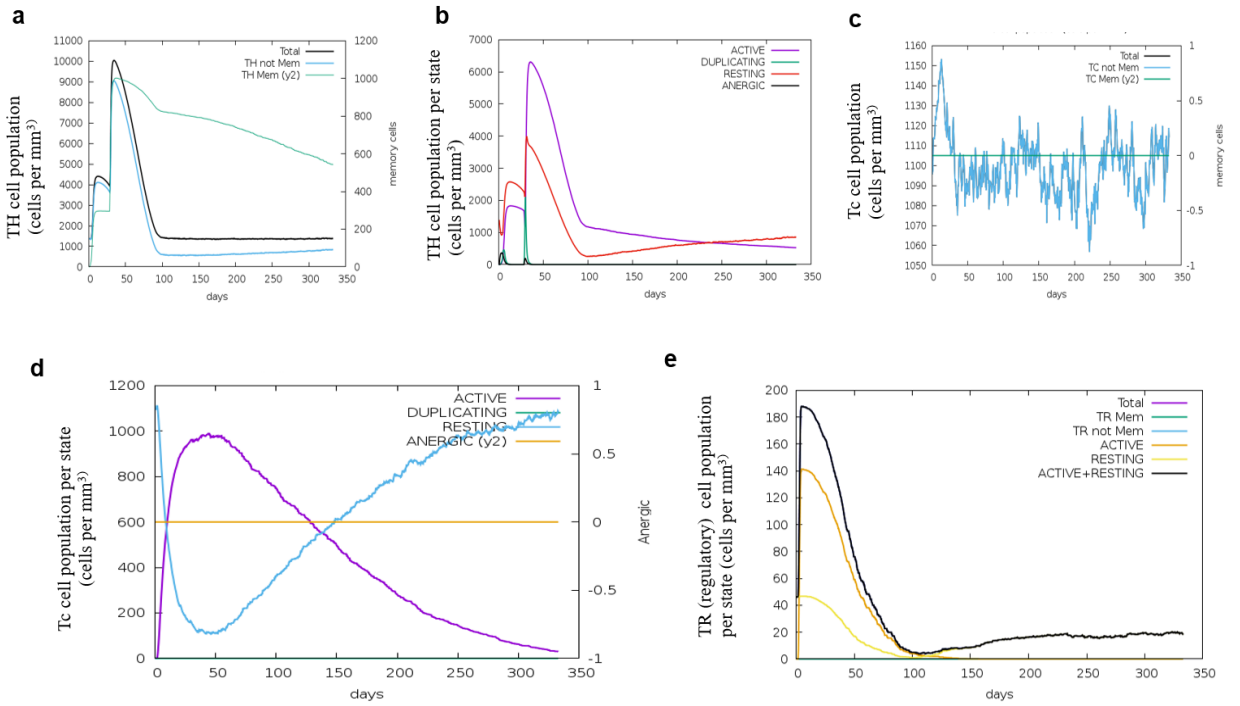

Figure 6. a) Helper T cell population b) Helper T cell population per state (cells per mm^3) c) TC cell population (cells per mm^3) d) TC cell population per state (cells per mm^3) e) TR cell population (cells per mm^3).

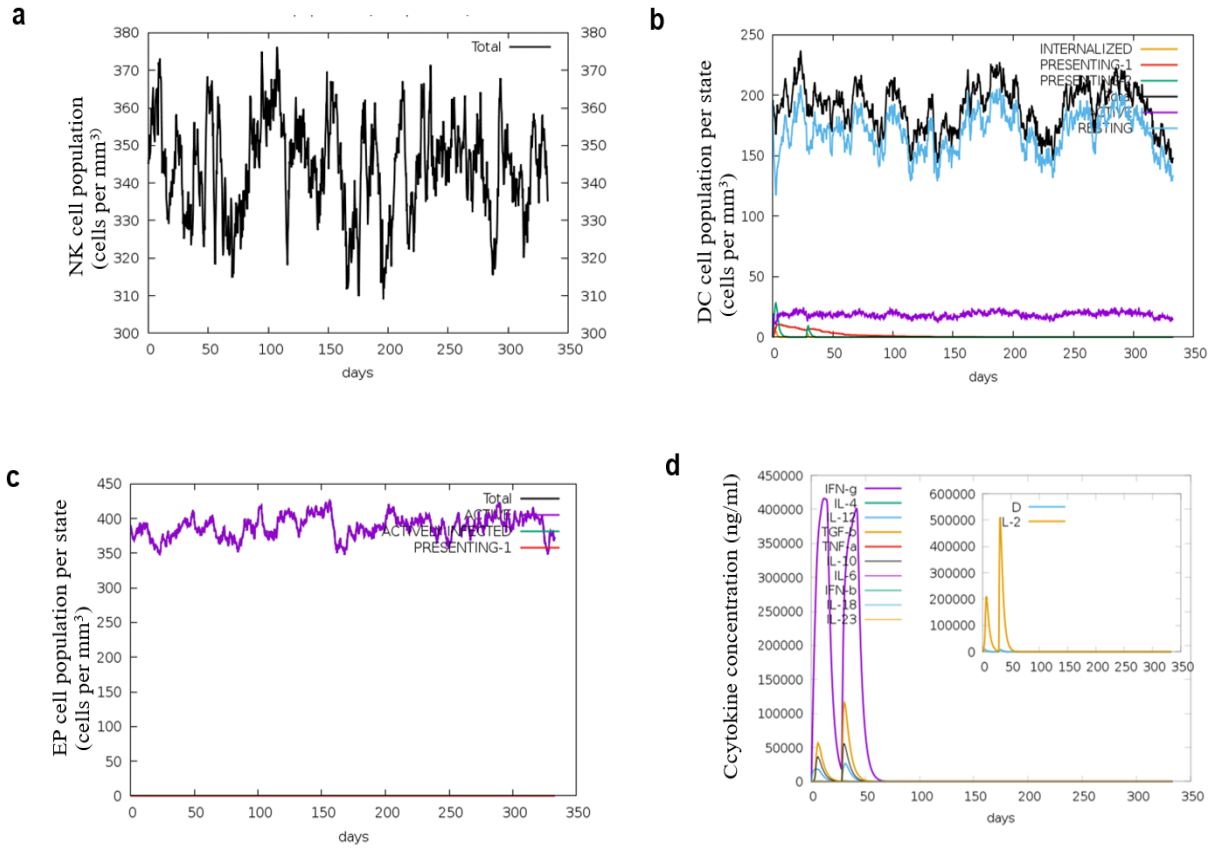

Figure 7. a) NK cell population b) Dendritic cell population c) Epithelial cell population per state d) Induction of cytokines and interleukins.

the refined vaccine candidate as -5.29 in comparison to the original model (z score: -4.53).

Molecular docking vaccine construct with TLR4s

Docking analysis showed that chains B and D of the TLR4 receptor formed polar interactions with the vaccine construct (Table 3).

Molecular dynamics simulation

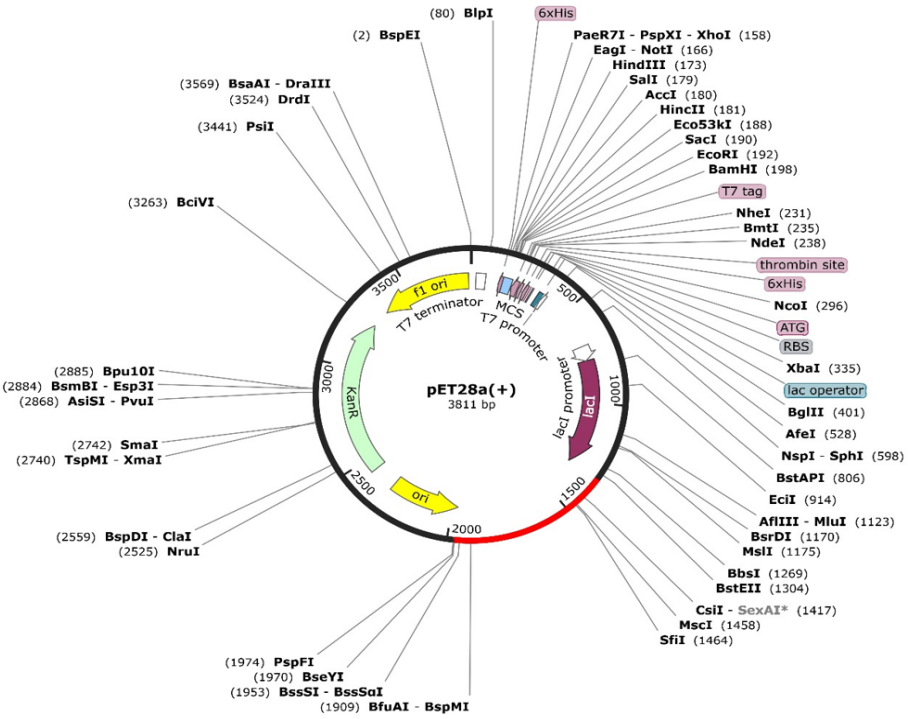

Figure 8. A Diagrammatic Map Generated for *in silico* GeneCloning in pET28(a)+ vector.

Table 3. Residues of Vaccine Forming Polar Interaction with *TLR4*

SN	TLR4 residues	Vaccine residues
1	Chain B: E605	H377
2	Chain-B: E603	K376
3	Chain-B: Q547	E366
4	Chain-B: Q523	R407
5	Chain-B: Y403	K412
6	Chain-B: E425	K415
7	Chain-D: E144	K390

The stability and compactness of the vaccine-TLR4 complex were assessed using the root mean square deviation (RMSD) and radius of gyration, respectively, as illustrated in Figure 4. The average RMSD was 0.92 ± 0.13 nm (Figure 4a), with oscillations below than 0.8 nm. Root mean square fluctuations (RMSF) were determined to track residue fluctuations over the 150 ns simulation (Figure 4b). The root mean square fluctuations were less in both chain B (atom numbers: 5825 to 11689) and chain D (atom numbers: 13078-14465) of TLR4 (RMSF-chain B: 0.17 ± 0.07 SD; chain D: 0.13 ± 0.05 SD) (Figure 4b). Similar results were obtained for the vaccine construct (atom numbers: 11689-18504) as well with a small fluctuation of 0.61 ± 0.0059 throughout the simulation. The average value of radius of gyration was found to be 4.27 ± 0.04 nm and remained steady after 30 ns, showing the compactness of the 3D protein structure during MD simulation (Figure 4c). SASA measures 839.9 ± 13.3 nm2, indicating that the hydrophobic core of vaccine and TLR4 complex is exposed to the aqueous environment (Figure 4d).

Characterisation of immune responses of the vaccine construct

The vaccine showed an increased level of IgM + IgG, along with IgG1 + IgG2, after antigenic exposure. The vaccine candidate induces cytokine production and activates antigen-presenting cells, such as macrophages, natural killer cells, regulatory T cells, memory B cells, dendritic cells, HTL, CTL, and epithelial cells. Epithelial cells play an important role in host defense and inflammation and are also involved in non-specific immunity. Overall, our vaccine induced both B- and T-cell-mediated immunity for approximately one year (350 days), as demonstrated by the graphical illustrations in Figure 5a-5d, 6a-6e and 7a-7d.

Codon adaptation

The length of the optimized codon was 1039 nucleotides, with a codon adaptation index (CAI) of 1.00, and a GC content of 47.88 (optimal range 30% to 70%). These results demonstrated that the expression of the vaccine was stable in the selected E. coli strain. Finally, an adapted gene sequence was inserted into the pET28a (+) vector to yield a recombinant plasmid (Figure 8).

Discussion

Breast cancer is the most common cancer, ranking first among all cancers in women, according to GLOBOCAN 2023. While the survival rate for breast cancer is relatively high (~90%), the mortality rate remains significant due to the high recurrence rates among affected women [24]. Immunotherapy currently plays a limited but significant role in the treatment of breast cancer, with high durability [24]. Recently, immunotherapeutic strategies, including checkpoint inhibitor therapies, monoclonal antibody-based treatments, and cancer vaccines, have significantly improved response rates in breast cancer patients. Breast cancer vaccines aim to target tumor-associated or tumor-specific antigens to stimulate the immune system. However, challenges remain in developing an effective vaccine for breast cancer, including selecting the optimal antigen targets, adjuvants, and immunization protocols [25]. Peptide-based vaccines are gaining attention due to their safety, ease of production, and the availability of several delivery modules. The peptides in peptide-based vaccines can be short (8-12 amino acids) or longer (≥ 20). Longer peptides are more stable and immunogenic than shorter peptides because they can enhance both humoral and cell-mediated immunity. Most clinical trials employ multiple epitopes, whereas in vitro studies generally rely on single peptides. The major advantage of multiple epitope-based vaccines is that they can overcome tumor immune escape [25]. Reverse vaccinology overcomes many drawbacks of conventional vaccine development. This approach has been utilized in the development of a vaccine for non-small cell lung cancer, where reverse vaccinology was employed to design a multi-epitope vaccine targeting cancer-testis antigens [26]. It can speed up the prediction of the most appropriate epitopes and therefore reduce time in the evaluation of the vaccine to 1-2 years [27].

In this study, we employed a reverse vaccinology approach combined with bioinformatics tools to design a multi-epitope vaccine against breast cancer that is antigenic, non-allergenic, non-toxic, stable, easy to purify for large-scale manufacturing, and immunogenic. We identified two protein candidates, AKT and *PARP1*, which possess all of these desired attributes. AKT, a member of the protein kinase family (A, G, and C), regulates a variety of cellular processes involved in tumorigenesis, such as proliferation, metastasis, and metabolism. The disrupted PI3K/Akt signaling pathway in breast cancer plays a crucial role in the disease's progression [28]. Poly (ADP-ribose) polymerase-1 (*PARP1*) is an enzyme that regulates transcription, cell cycle progression, tumor formation, and DNA damage. Upregulation of *PARP1* in breast tumors signify the discovery of *PARP1* inhibitors in BC [29, 30]. Multi-epitope vaccines are constructed by identifying tumor antigens, protein structure analysis, T and B cell epitope prediction, epitope characterization, and protein-epitope interaction evaluation [31]. For vaccine development, predicting B-cell and T-cell epitopes is crucial, as it enhances immunogenicity by promoting the production of antigen-specific antibodies and activating CD4+ (helper T cells) and CD8+ (cytotoxic T cells).

These CD4+ and CD8+ cells, along with an adjuvant, are essential components of a peptide vaccine and play a key role in the design of a multi-epitope peptide-based vaccine [32]. Although epitope-based peptide vaccines possess limited immunogenicity, adjuvants can be used as a solution [31]. To boost the immune response, a TLR4 agonist was used as an adjuvant, which was linked to B- and T-cell epitopes using three linkers. Toll-like receptor (TLR) agonists have been found to be efficient vaccine adjuvants with anticancer properties. TLR's play a vital role in bridging innate and adaptive immune responses in cancer [33]. In this study, the adjuvant used was the 50S ribosomal L7/L12 protein (Accession No.: P9WHE3), a TLR-4 agonist known to stimulate a strong immune response. Toll-like receptor (TLR-4) interacts with innate immune cells, such as macrophages, dendritic cells (DCs), and natural killer (NK) cells, and is crucial in initiating immune responses. TLR-4 is expressed not only in immune cells but also in tumor cells, where it plays a significant role in promoting antitumor activity within the tumor microenvironment [34-36]. Molecular dynamics simulation (MDS), a computational technique, was employed to investigate the physical interactions between the vaccine and TLR4 within a biophysical environment. This approach enabled the assessment of structural changes and the flexibility of the docked complex. The study revealed a strong interaction between the vaccine and TLR4, with minimal fluctuations and high stability throughout the 150 ns MD simulation. Additionally, the vaccine construct was predicted to trigger an immune response upon antigen injection, as forecasted by the C-IMMSIM server.

Our findings suggest that the designed vaccine holds promise for treating breast cancer; however, relying solely on computational approaches is insufficient and have limitations. To enhance the practical feasibility of our vaccine, it must be validated through appropriate in vitro and animal models. If the vaccine proves effective in these models, further clinical trials can be conducted on patients to assess its safety, toxicity, and immune response profile in individuals with breast cancer. The future potential of these vaccines lies in the development of multi-target vaccines, improved stability, and simplified production processes. Multi-target vaccines are promising, offering the potential to enhance immune responses by targeting multiple antigens simultaneously. This approach can improve the vaccine's effectiveness, reduce the likelihood of immune escape, and provide broader protection against diverse strains or variations of a disease.

In conclusion, breast cancer remains a significant global health issue, and there is an urgent need to improve treatment options. Current therapies, including chemotherapy, surgery, and vaccines, often have limited effectiveness and come with various side effects. In this study, we focused on two proteins, *AKT1* and *PARP1*, as potential candidates for a vaccine against breast cancer. We designed a multi-epitope vaccine by incorporating several B and T cell epitopes, along with a TLR4 agonist as an adjuvant. The vaccine demonstrated strong binding affinity for TLR4 chains B and D. Immune simulations revealed elevated levels of IgM, IgG1, and IgG2, along with reduced antigen levels, while no IgE synthesis was observed, preventing any allergic immune response. The vaccine also showed favorable physicochemical properties, making it suitable for manufacturing, purification, formulation, and storage. These findings suggest that this vaccine could be a promising option for breast cancer treatment. However, further preclinical validation, optimization, and thorough assessment of its safety, efficacy, and potential side effects are needed to fully evaluate its therapeutic potential.

Author Contribution Statement

Sakshi Gupta: Conceptualization, Methodology, Data analysis, writing an original draft. Mansi Patel, Drishti Desai, Daksh Kunchala, Suresh Prajapati: Data analysis and validation. Charmie Jyotishi: made figure 1 and revised the manuscript, Reeshu Gupta: Conceptualization, supervision, review the original draft, and finalizing the draft. .

Acknowledgements

This study is supported via funding from Prince Sattam Bin Abdulaziz University project number (PSAU/2024/R/1445).

Funding

This research receives funding from Prince Sattam Bin Abdulaziz University project number (PSAU/2024/R/1445) and Parul University (CR4D/IMSL/084)..

Data availability

All data used in this study were publicly accessible. The analyzed data and code that support the findings presented in this study are available from the corresponding author upon reasonable request.

Ethical approval

The study was approved by the Ethics Committee of Parul University (PUIECHR/ PIMSR/00/081734/5308).

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Burguin A, Diorio C, Durocher F. Breast cancer treatments: Updates and new challenges. J Pers Med. 2021;11(8):808. https://doi.org/10.3390/jpm11080808.
2. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against her2 for metastatic breast cancer that overexpresses her2. N Engl J Med. 2001;344(11):783-92. https://doi.org/10.1056/NEJM200103153441101.
3. Capelan M, Pugliano L, De Azambuja E, Bozovic I, Saini KS, Sotiriou C, et al. Pertuzumab: New hope for patients with her2-positive breast cancer. Ann Oncol. 2013;24(2):273-82.

https://doi.org/10.1093/annonc/mds328.
4. Mittendorf EA, Clifton GT, Holmes JP, Schneble E, van Echo D, Ponniah S, et al. Final report of the phase i/ii clinical trial of the e75 (nelipepimut-s) vaccine with booster inoculations to prevent disease recurrence in high-risk breast cancer patients. Ann Oncol. 2014;25(9):1735-42. https://doi.org/10.1093/annonc/mdu211.
5. Al-Hawary SIS, Saleh EAM, Mamajanov NA, N SG, Alsaab HO, Alghamdi A, et al. Breast cancer vaccines; a comprehensive and updated review. Pathol Res Pract. 2023;249:154735. https://doi.org/10.1016/j.prp.2023.154735.
6. Gambirasi M, Safa A, Vruzhaj I, Giacomin A, Sartor F, Toffoli G. Oral administration of cancer vaccines: Challenges and future perspectives. Vaccines (Basel). 2023;12(1):26. https://doi.org/10.3390/vaccines12010026.
7. Singh P, Shaikh S, Gupta S, Gupta R. In-silico development of multi-epitope subunit vaccine against lymphatic filariasis. J Biomol Struct Dyn. 2023:1-15. https://doi.org/10.1080/07391102.2023.2294838.
8. Dimitrov I, Bangov I, Flower DR, Doytchinova I. Allertop v.2--a server for in silico prediction of allergens. J Mol Model. 2014;20(6):2278. https://doi.org/10.1007/s00894-014-2278-5.
9. Goodman RE, Ebisawa M, Ferreira F, Sampson HA, van Ree R, Vieths S, et al. Allergenonline: A peer-reviewed, curated allergen database to assess novel food proteins for potential cross-reactivity. Mol Nutr Food Res. 2016;60(5):1183-98. https://doi.org/10.1002/mnfr.201500769.
10. Yin L, Gupta R, Vaught L, Grosche A, Okunieff P, Vidyasagar S. An amino acid-based oral rehydration solution (aa-ors) enhanced intestinal epithelial proliferation in mice exposed to radiation. Sci Rep. 2016;6:37220. https://doi.org/10.1038/srep37220.
11. Tran AN, Dussaq AM, Kennell T, Jr., Willey CD, Hjelmeland AB. Hpaanalyze: An r package that facilitates the retrieval and analysis of the human protein atlas data. BMC Bioinformatics. 2019;20(1):463. https://doi.org/10.1186/s12859-019-3059-z.
12. El-Manzalawy Y, Dobbs D, Honavar V. Predicting linear b-cell epitopes using string kernels. J Mol Recognit. 2008;21(4):243-55. https://doi.org/10.1002/jmr.893.
13. Reynisson B, Alvarez B, Paul S, Peters B, Nielsen M. Netmhcpan-4.1 and netmhciipan-4.0: Improved predictions of mhc antigen presentation by concurrent motif deconvolution and integration of ms mhc eluted ligand data. Nucleic Acids Res. 2020;48(W1):W449-W54. https://doi.org/10.1093/nar/gkaa379.
14. Peters B, Sette A. Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. BMC Bioinformatics. 2005;6:132. https://doi.org/10.1186/1471-2105-6-132.
15. Nagpal G, Usmani SS, Dhanda SK, Kaur H, Singh S, Sharma M, et al. Computer-aided designing of immunosuppressive peptides based on il-10 inducing potential. Sci Rep. 2017;7:42851. https://doi.org/10.1038/srep42851.
16. Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, Thornton JM. Aqua and procheck-nmr: Programs for checking the quality of protein structures solved by nmr. J Biomol NMR. 1996;8(4):477-86. https://doi.org/10.1007/BF00228148.
17. Comeau SR, Gatchell DW, Vajda S, Camacho CJ. Cluspro: A fully automated algorithm for protein-protein docking. Nucleic Acids Res. 2004;32(Web Server issue):W96-9. https://doi.org/10.1093/nar/gkh354.
18. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. Ucsf chimera--a visualization system for exploratory research and analysis. J Comput Chem. 2004;25(13):1605-12. https://doi.org/10.1002/jcc.20084.
19. Yan Y, Tao H, He J, Huang SY. The hdock server for integrated protein-protein docking. Nat Protoc. 2020;15(5):1829-52. https://doi.org/10.1038/s41596-020-0312-x.
20. Kanse S, Khandelwal M, Pandey RK, Khokhar M, Desai N, Kumbhar BV. Designing a multi-epitope subunit vaccine against vp1 major coat protein of jc polyomavirus. Vaccines (Basel). 2023;11(7):1182. https://doi.org/10.3390/vaccines11071182.
21. Rapin N, Lund O, Bernaschi M, Castiglione F. Computational immunology meets bioinformatics: The use of prediction tools for molecular binding in the simulation of the immune system. PLoS One. 2010;5(4):e9862. https://doi.org/10.1371/journal.pone.0009862.
22. Grote A, Hiller K, Scheer M, Munch R, Nortemann B, Hempel DC, et al. Jcat: A novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 2005;33(Web Server issue):W526-31. https://doi.org/10.1093/nar/gki376.
23. Dennis KL, Blatner NR, Gounari F, Khazaie K. Current status of interleukin-10 and regulatory t-cells in cancer. Curr Opin Oncol. 2013;25(6):637-45. https://doi.org/10.1097/CCO.0000000000000006.
24. Tokumaru Y, Joyce D, Takabe K. Current status and limitations of immunotherapy for breast cancer. Surgery. 2020;167(3):628-30. https://doi.org/10.1016/j.surg.2019.09.018.
25. Abd-Aziz N, Poh CL. Development of peptide-based vaccines for cancer. J Oncol. 2022;2022:9749363. https://doi.org/10.1155/2022/9749363.
26. Herrera LRM. Reverse vaccinology approach in constructing a multi-epitope vaccine against cancer-testis antigens expressed in non-small cell lung cancer. Asian Pac J Cancer Prev. 2021;22(5):1495-506. https://doi.org/10.31557/APJCP.2021.22.5.1495.
27. Kanampalliwar AM. Reverse vaccinology and its applications. Methods Mol Biol. 2020;2131:1-16. https://doi.org/10.1007/978-1-0716-0389-5_1.
28. Basu A, Lambring CB. Akt isoforms: A family affair in breast cancer. Cancers (Basel). 2021;13(14). https://doi.org/10.3390/cancers13143445.
29. Ossovskaya V, Koo IC, Kaldjian EP, Alvares C, Sherman BM. Upregulation of poly (adp-ribose) polymerase-1 (parp1) in triple-negative breast cancer and other primary human tumor types. Genes Cancer. 2010;1(8):812-21. https://doi.org/10.1177/1947601910383418.
30. Tung N, Garber JE. Parp inhibition in breast cancer: Progress made and future hopes. NPJ Breast Cancer. 2022;8(1):47. https://doi.org/10.1038/s41523-022-00411-3.
31. Prawiningrum AF, Paramita RI, Panigoro SS. Immunoinformatics approach for epitope-based vaccine design: Key steps for breast cancer vaccine. Diagnostics (Basel). 2022;12(12). https://doi.org/10.3390/diagnostics12122981.
32. Nezafat N, Ghasemi Y, Javadi G, Khoshnoud MJ, Omidinia E. A novel multi-epitope peptide vaccine against cancer: An in silico approach. J Theor Biol. 2014;349:121-34. https://doi.org/10.1016/j.jtbi.2014.01.018.
33. Yusuf N. Toll-like receptor mediated regulation of breast cancer: A case of mixed blessings. Front Immunol. 2014;5:224. https://doi.org/10.3389/fimmu.2014.00224.
34. Li J, Yang F, Wei F, Ren X. The role of toll-like receptor 4 in tumor microenvironment. Oncotarget. 2017;8(39):66656-67. https://doi.org/10.18632/oncotarget.19105.
35. Papadakos SP, Arvanitakis K, Stergiou IE, Lekakis V, Davakis S, Christodoulou MI, et al. The role of tlr4 in the immunotherapy of hepatocellular carcinoma: Can we teach

an old dog new tricks? Cancers (Basel). 2023;15(10). https://doi.org/10.3390/cancers15102795.
36. Madanagopal P, Muthusamy S, Pradhan SN, Prince PR. Construction and validation of a multi-epitope in silico vaccine model for lymphatic filariasis by targeting brugia malayi: A reverse vaccinology approach. Bull Natl Res Cent. 2023;47(1):47. https://doi.org/10.1186/s42269-023-01013-0