



# Immunoinformatics studies and design of breast cancer multiepitope peptide vaccines: Diversity analysis approach

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## ABSTRACT

Breast cancer is one of the most diagnosed cancers in women; the number of cases continues to rise. The high prevalence and increased incidence need more attention in developing effective therapy. Current passive therapy has several drawbacks that have not yet been resolved. Thus, an alternative and preventive therapy for cancer is needed by utilizing vaccines. Immunoinformatics approach is one of the promising methods predicting epitopes in vaccine research. This approach could accelerate the initial study process of vaccine development and reduce research costs. Epitope conservancy and vaccine coverage are important parameters in vaccine research due to addressing the variability and diversity of cancer genomics. This study will be carried out on the multiepitope characterization of potential T cells against the protein mechanism in breast cancer. Proteins used in this study are Mucin-4, Phosphatase And Tensin Homolog, and Receptor tyrosine-protein kinase erbB-2. CTL epitopes, antigenicity, immunogenicity, allergenicity, and toxicity were predicted for the peptide vaccine. Immunoinformatics analysis generates a multiepitope sequence consisting of seven epitopes: DPVALVAPF, SVAYRLGTL, SQINTLNLT, RFRELVSEF, VTSANIQEF, RPRFRELVS, and MYFEFPQPL by AAY linker. The docking and molecular dynamics analyses were conducted to confirm the interactions between the multiepitope vaccine molecule and TLR-4-MD. The multiepitope vaccine construct can be an appropriate choice for further experiments.

## INTRODUCTION

Cancer is a disease caused by abnormal cells in the body's tissues that grow and develop rapidly and become uncontrolled. This disease is known as one of the world's major public health problems causing morbidity and mortality, and the number of cancer cases reaches more than 19 million (Ferlay *et al.*, 2020). In 2012, the most diagnosed cancer in women was breast cancer (43.3%) and cervical cancer (14%) (Tao *et al.*, 2015). Compared to 2012, the number of cases increased by 24% in 2018. This number is expected to continue to rise (Bray *et al.*, 2018). The high prevalence and increased incidence made urgent research for effective therapies for cancer (Ghoncheh *et al.*, 2016; Parvizpour *et al.*, 2018).

Currently, treatment of some types of breast cancer was conducted using monoclonal antibodies against HER2 (Atapour *et al.*, 2020). The limitation of passive therapy such as high cost, treatment duration, and the possibility of emerging immunological tolerance has not been resolved (Pallerla *et al.*, 2021). As an alternative to monoclonal antibody therapy with a limited clinical benefit over time, vaccines stimulating immune response toward tumor cells are one of the therapeutic strategies as a potential approach for cancer therapy. Active immunotherapy promoted by the tumor-associated antigen vaccine or tumor-specific antigen will increase preexisting resistance to antigens and prolonged activation of the immune system (Benedetti *et al.*, 2017).

Promising vaccine development methods in the genomic era are by using the reverse vaccinology method using bioinformatics and immunoinformatics approach. This reverse vaccinology approach predicts epitopes, an antigen determinant that has an important role in immunity, and its interaction toward human leukocyte antigen (HLA). Through this approach, vaccine development can be carried out based on the target sequence and genomic data without culturing the target organism

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(Kanamalliwar *et al.*, 2013). Also, this approach was able to accelerate the initial study process of vaccine development and reduce research costs. The predicted protein from this *in silico* study can then be synthesized for testing on test animals (Khan *et al.*, 2006; Serruto and Rappuoli, 2006).

Cancer prevalence, incidence, and mortality between diverse ethnic populations are very different. The causes for these differences are complex, including intrinsic factors (genetic variation) and extrinsic factors (social, economic, and geographical) (Khan *et al.*, 2017). Understanding cancer risk and the underlying cause is essential in developing research and health care practices that could work for various ethnicities and populations. Therefore, in the vaccine development process, it is important to pay attention to vaccine coverage so that vaccines can cover various ethnicities.

Some proteins are overexpressed in cancer patients and associated with enhanced tumor growth. These overexpressed proteins are the subject of active immunotherapy in vaccination. In this study, we use protein Receptor tyrosine-protein kinase erbB-2 (ERBB2), Mucin-4 (MUC4), and Phosphatase And Tensin Homolog (PTEN) for vaccine development. ERBB2 is a gene encoding growth factor receptor that plays a significant role in breast cancer (Ludovini *et al.*, 2008). ERBB2 is often overexpressed in breast cancer and has been considered as a potential target in cancer therapeutics (Chentoufi *et al.*, 2009). MUC4 is membrane-bound mucin expressed in several epithelial malignancies. Mucin is normally expressed by epithelial cells as a barrier and contributes to lubrication. MUC4 is mucin that is apparently specific to tumor tissue. Recent studies showed that MUC4 differentially expressed in breast cancer cells appears to correlate with prognosis (Hatrup and Gendler, 2008). PTEN protein is involved in the regulation of several crucial cell functions. Partial loss of PTEN function is enough to promote tumorigenesis and accelerate cancer progression (Bazzichetto *et al.*, 2019). Mutations in these proteins lead to the variability in sequence composition. Conserved sequence regions are critical to addressing the diversity of the proteins (Khan *et al.*, 2008).

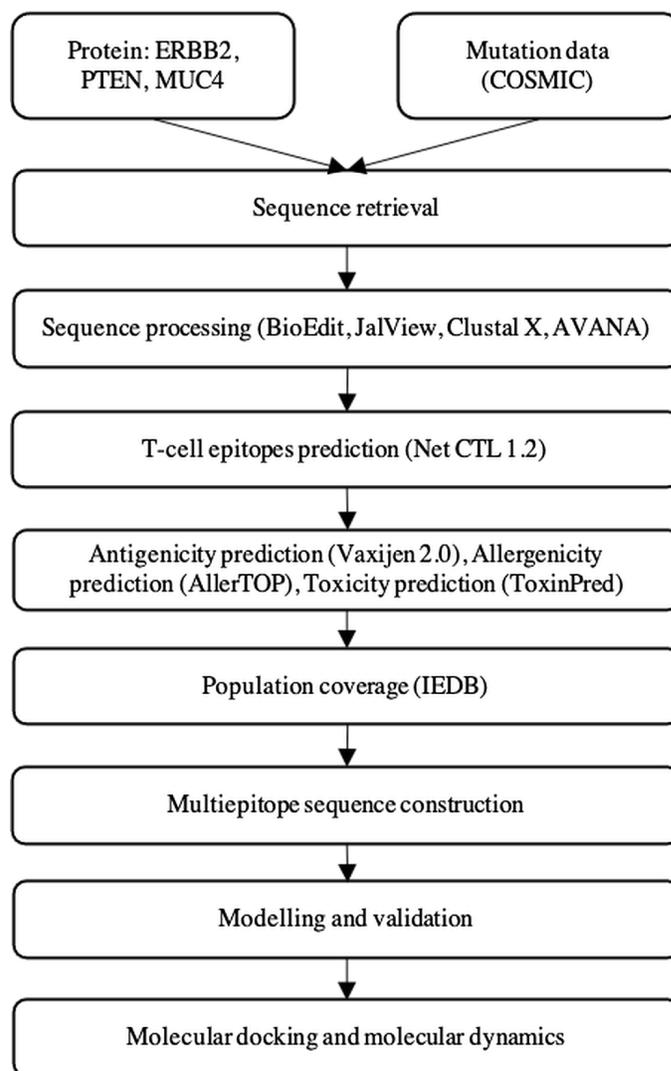
This study will be carried out on multipeptide characterization of potential T cells against protein mechanisms in breast cancer, diversity analysis of protein sequences related to immunity, effects of antigenic variation, and sequence similarity to the immune response, HLA. The urgency of this study is based on the high incidence of breast cancer in Indonesia and breast cancer drug resistance. Another alternative is needed to prevent and treat breast cancer by utilization of vaccines.

## METHODS

The methodology used in this study is summarized in a diagram (Fig. 1).

### Data retrieval

ERBB2 (P04626), PTEN (P60484), and MUC4 (Q99102) protein sequences were retrieved from the UniProt web server (<https://www.uniprot.org/>) (Bateman, 2019). Mutation data and ERBB2, PTEN, and MUC4 protein sequences were downloaded from the COSMIC website (<https://cancer.sanger.ac.uk/cosmic>) (Tate *et al.*, 2019). The mutation data downloaded



**Figure 1.** The methodology of the research.

are the missense type mutation data. The mutations were induced in the retrieved sequence by BioEdit (Hall, 1999).

### Sequence processing and diversity analysis

After obtaining a sequence with a mutation according to COSMIC data, the sequence is then processed through the JalView software to delete the twin sequences so that the sequences obtained are unique (Waterhouse *et al.*, 2009). The sequences were then aligned using the ClustalX software (Larkin *et al.*, 2007). Mutation in this protein appears to be associated with developing breast cancer. As the gene has a high mutation number based on the database, it is important to search regions of protein which are conserved. The sequences were then analyzed for entropy using the AVANA software to determine the conserved region.

### Prediction of the epitope and its characteristics

T-cell epitope prediction was carried out on the NetCTL 1.2 page (<https://services.healthtech.dtu.dk/service.php?NetCTL-1.2>) based on 12 HLA class I supertypes (A1, A2, A3, A24, B7, B8, B27, B44, B58, B62, C1, and C4) (Larsen *et al.*,

2007). The prediction results with NetCTL 1.2 will provide data on the affinity value of HLA class I and C terminal binding, the value of transport effectiveness via TAP, and the total affinity value (Grifoni *et al.*, 2020). The epitope chosen is an epitope with a value above 0.75. Epitopes obtained from the NetCTL page predicted their immunogenicity characteristics through the IEDB Immunogenicity page (<http://tools.iedb.org/immunogenicity/>) (Calis *et al.*, 2013).

Immunogenicity and antigenicity are important parameters in vaccine design. Therefore, epitopes that have been obtained from the predictions via the NetCTL 1.2 were submitted to the IEDB immunogenicity page. Epitopes with positive immunogenicity values were further predicted for their antigenicity characteristics through the VaxiJen 2.0 page (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) (Doytchinova and Flower, 2007), allergenic characteristics on the AllerTOP page (<https://www.ddg-pharmfac.net/AllerTOP/>) (Dimitrov *et al.*, 2013), and the characteristics of the toxicity on the ToxinPred page (<http://www.imtech.res.in/raghava/toxinpred/>) (Gupta *et al.*, 2013). VaxiJen and AllerTOP, respectively, identify the antigenicity and allergenicity of epitopes based on physicochemical properties of proteins (Dimitrov *et al.*, 2013; Doytchinova and Flower, 2007). In the antigenicity prediction test using VaxiJen 2.0, 0.5 was kept as a threshold score and epitopes with a higher score were considered for further analysis. Toxicity prediction by ToxinPred was based on support vector machine from toxic peptides dataset (Joshi *et al.*, 2020). ToxinPred prediction consists of two steps, toxicity prediction and analog generation and prediction (Gupta *et al.*, 2013). Epitopes selected as candidates are epitopes with an antigenicity value of more than 0.5 and nontoxic and nonallergen characteristics.

### Epitope population coverage prediction

The coverage of the CTL epitope population is predicted via the IEDB Population Coverage page with default parameters, based on Major Histocompatibility Complex (MHC) binding restriction data (<http://tools.iedb.org/population/>). This dataset consists of frequencies for 115 countries, 21 ethnicities, and 16 geographical areas. Each CTL epitope is inserted to that page along with the HLA type data that binds to the epitope. After that, select the area and population to predicts epitope population coverage. The areas selected are world, East Asia, Northeast Asia, South Asia, Southeast Asia, Southwest Asia, Europe, Central Africa, East Africa, North Africa, South Africa, West Africa, West Indies, Central America, North America, South America, and Oceania (Bui *et al.*, 2006).

### Multiepitope sequence construction

Each of the selected epitopes was then linked with AAY linker from the study by Klein *et al.* (2014) and a HEYGAEALERAG motif was added to enhance epitope presentation (Tani *et al.*, 2000).

### Epitope modeling

3D modeling of the putative multiepitope vaccine was conducted through Swiss-PdbViewer/DeepView (Guex and Peitsch, 1997). The residues of the structure can be seen through the Control Panel menu. The attachment of the vaccine structure to the template is done through the Magic Fit menu.

The adhered residues can be seen through the alignment menu. Visualization of the tertiary structure of the adhered vaccine is done by removing the tertiary structure of the template so that the tertiary structure of the vaccine can be seen. The improvement of the tertiary structure of the vaccine is carried out in two stages. The first step is to improve the structure of the vaccine in the DeepView viewer by fixing the overlapping residues, whereas the second stage is through the Swiss-Model server by sending the structure of each vaccine that has been upgraded on DeepView to the Swiss-Model server in the form of PDB for structure optimization, namely, by selecting the Optimize Mode menu on the Swiss-Model server.

### Validation of tertiary structure

The evaluation of tertiary structure of putative multiepitope vaccine was done using Ramachandran plot in DeepView program. Some parameters were used to evaluate the quality of 3D model of putative multiepitope such as MolProbability score, Ramachandran favored, Ramachandran outlier, rotamer outlier value, clash score, C beta deviation, bad bonds, and bad angels (Johansson *et al.*, 2012).

### Molecular docking

The initial stage of the molecular docking simulation is 3D modeling of the selected multiepitope sequence. 3D modeling is carried out on the SWISS-MODEL page (<https://swissmodel.expasy.org>) and assessment for the 3D structure using Ramachandran plot (Gopalakrishnan *et al.*, 2007; Waterhouse *et al.*, 2018). The macromolecule TLR4 was downloaded from the Protein Data Bank (<https://www.rcsb.org/>) with PDB ID 4R7D (Loyau *et al.*, 2015). Molecular docking was conducted using protein-protein docking in Rosetta web server (<https://rosie.graylab.jhu.edu/docking2>) (Chaudhury *et al.*, 2011; Lyskov and Gray, 2008; Lyskov *et al.*, 2013). PyMOL software was used for visualization and amino acid interaction evaluation for molecular docking results (Delano, 2002).

### Molecular dynamic simulation

The molecular dynamic simulation was executed on the LARMD website (<http://chemyang.cnu.edu.cn/ccb/server/LARMD>) (Yang *et al.*, 2020). LARMD is developed based on Amber16 software (Case *et al.*, 2016). Molecular dynamic simulation has workflow as follows: preparation complex protein-ligand, force field generation, minimization, equilibration, production, and result analysis root-mean-square deviation [RMSD], root-mean-square fluctuation [RMSF], B-factor, fraction of native contact, and radius of gyration [Rg] (Best *et al.*, 2013; Chovancova *et al.*, 2012; Grant *et al.*, 2006; McGibbon *et al.*, 2015).

## RESULTS AND DISCUSSION

### Sequence processing

Bioinformatics and immunoinformatics have been supporting large amounts of biological data in vaccine research. Several tools in this approach have been developed to process sequence and analyze immunological data (Raman *et al.*, 2014). JalView is used to remove identical sequences and generate

unique sequences. These unique sequences are aligned using ClustalX. Alignment files are converted into \*.aln files by BioEdit. The processed sequence then being analyzed its entropy using AVANA, a software based on the Shannon Entropy concept. Shannon sequence entropy analysis measures the conservation level and variability of the sequence of certain lengths. Conserved peptides with lower entropy scores are more conserved and less random than peptides with higher entropy (Li *et al.*, 2019). The conserved 9-mer peptides from AVANA are then analyzed further for the epitope prediction (Khan *et al.*, 2006).

### Prediction of T-cell epitopes

The conserved peptides are used for T cell putative epitopes identification using an immunoinformatics approach. Putative epitopes chosen for further analysis should be antigenic and immunogenic. Antigenicity is the ability of a peptide to bind and interact specifically with the functional binding site of an antibody. Immunogenicity refers to the ability of the peptide to induce an immune response (Choudhuri, 2014).

One of the important stages in peptide-based vaccine development is the prediction of antigenic epitopes that potentially bind to different HLA alleles. Recognition of putative epitopes by T-cell receptor (TCR) will induce an immune response. Putative epitopes that are recognized by TCR will be presented on the surface of Antigen Presenting Cells bound to MHC. There are two types of MHC: MHC Class I (HLA class I) and MHC Class II (HLA class II). MHC or HLA molecules are polymorphic and thousands of HLA are identified. Therefore, in putative epitope vaccine design, selection of putative epitopes that bind to a larger number of HLA supertypes could result in increased population coverage (Patronov and Doytchinova, 2013).

The prediction of T-cell putative epitopes was conducted by data-driven using NetCTL-1.2 tools for CTL (HLA I) putative epitopes. The NetCTL-1.2 tools integrate prediction of peptide MHC Class I binding, proteasomal C terminal cleavage, and TAP transport efficiency for 12 HLA supertypes (Larsen *et al.*, 2007). The peptide that has a proteasomal recognition site is not favorable as a putative epitope because it will undergo degradation during processing. TAP binding and transport efficiency are important because the peptides must be transported by TAP to be presented on MHC Class I. The higher the score, the higher the possibility for the putative epitopes to be transported by TAP (Bhasin, 2004).

Epitopes are obtained from the NetCTL server and then its antigenicity is evaluated using VaxiJen and immunogenicity using IEDB. Antigenicity and immunogenicity are important parameters for vaccine development to ensure that the peptide sequence could induce an immune response (Kalita *et al.*, 2020). Besides that, the peptide sequence has to be nontoxic and nonallergenic (Dimitrov *et al.*, 2013).

The nonantigenic putative epitopes were removed, subjected to predict its toxicity and allergenicity. Toxic and allergenic putative epitopes were removed. The results of the CTL epitope analysis, antigenicity, and immunogenicity of the three proteins are summarized in Table 1. The analysis was also conducted for an epitope-based vaccine which has been evaluated through the clinical phase, such as E75 and GP2 vaccine with amino acid composition, respectively, KIFGSLAFL and IISAVVGIL. Both of these vaccines induce CD8+ CTL response (Brown *et al.*, 2020).

Despite the fact that extensive research has been done in epitope-based peptide-vaccine development, the vaccine has not yet been approved for human use. This is due to the low immunogenicity of the simple peptide vaccine and its HLA-restricted characteristics. Hence, it is necessary to construct a multiepitope vaccine targeting different HLAs. In addition, the utilization of adjuvants or certain motifs is used to enhance the immunogenicity of the vaccine (Purcell *et al.*, 2007; Topuzoğullari *et al.*, 2020). Based on the results of the CTL epitope prediction in Table 1, a multiepitope sequence is constructed as a vaccine candidate, with an arrangement as shown in Figure 2. Each epitope is connected to an AAY linker and added to a motif HEYGAEALERAG at the N end to enhance epitope presentation

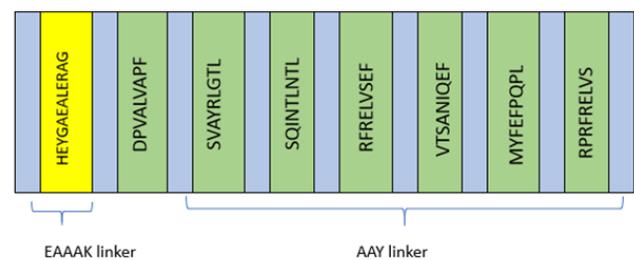


Figure 2. Multiepitope vaccine constructs.

Table 1. Putative epitope prediction results.

Protein	Epitope CTL	HLA supertypes	Immunogenicity	Antigenicity
	RFRELVSEF	B8, B62, and B39	0.06449	1,1717
ERBB2	VTSANIQEF	A1, A24, B58, and B62	0,06539	0,7462
	RPRFRELVS	B7 and B8	0.29401	1,3619
PTEN	MYFEFPQPL	A24 and B39	0,1381	1,285
	DPVALVAPF	A1 and B39	0,10737	0,92
MUC4	SVAYRLGTL	B39 and B58	0,10022	0,8354
	SQINTLNTL	A1, B27, and B58	0,08127	1,4349
Positive control	KIFGSLAFL		0.00198	Nonantigen
	IISAVVGIL		0.17109	Nonantigen

(Safavi *et al.*, 2019). The motif and epitopes were linked by the EAAAK linker.

### Epitope population coverage prediction

MHC or HLA molecules are polymorphic and more than thousands of HLA are identified. In humans, more than 225 HLA Class I and 980 HLA Class II alleles have been identified. The regional polymorphisms encoding the peptide-binding pathways of the HLA molecule give MHC molecules varying binding specificities. In addition, the frequency of allele variants varies among different ethnicities. It would be complex if different epitopes were produced to cover different ethnicities or regions (Sakib *et al.*, 2014). Therefore, it is important to select a putative epitope with a broad and maximal population coverage in addition to the ability to bind HLA.

IEDB population coverage tools generate a prediction of population coverage from the vaccine construct for all regions available and the world, as presented in Figure 3. From the graphic, the vaccine constructs showed good percentage coverage for all regions/populations available in IEDB webserver. Maximum coverage (83.72%) was found in the population of Oceania. Population coverage from the world population was 76.59%. All of the percentage coverage was above 55%; only one population, Central America population, has 2.18% population coverage. The small population coverage was in the Central America region because the Central America region provided scarce information from very specific populations (Michel-Todó *et al.*, 2019).

### 3D molecular prediction of putative multi-epitope vaccine

The homology approach was used in research for the tertiary structure predictions of vaccines. The tertiary structure prediction of a protein with homology modeling can be done in terms of the similarity of the sequence between the protein and the database protein which is at least 20% (Bourne, 2003). The results of the homology modeling sequence of the multi-epitope vaccine with the protein in the database show that it is greater than 50%, so the prediction of the vaccine structure can be done using the homology modeling method.

Visualization of the tertiary structure of the reverse immunization vaccine is shown in Figure 4A. The vaccine design has similar sequences to the TLR4 proteins, which are expected to have the same function and trigger an immune response as in TLR4. Improvements to the tertiary structure of vaccines need to be done to repair residues that overlap each other. Proteins that contain the same amino acid structure as the template do not necessarily fold (folding) proteins in their tertiary form because of the influence of interactions between amino acids with side amino acids or neighboring amino acids. The 3D structure validation is carried out with the Ramachandran plot as shown in Figure 4B.

The glycine plot can be in the disallowed region because glycine does not have side chains, so the angle  $\phi$  (phi) and angle  $\psi$  (psi) are infinite. The number of residual plots other than glycine in the restricted area indicates the structural quality of the protein. If the amount exceeds 15% of the total protein residue, the protein quality is poor (Cherkasov, 2005). The amount of nonglycine residue in the disallowed region on the Ramachandran plot for

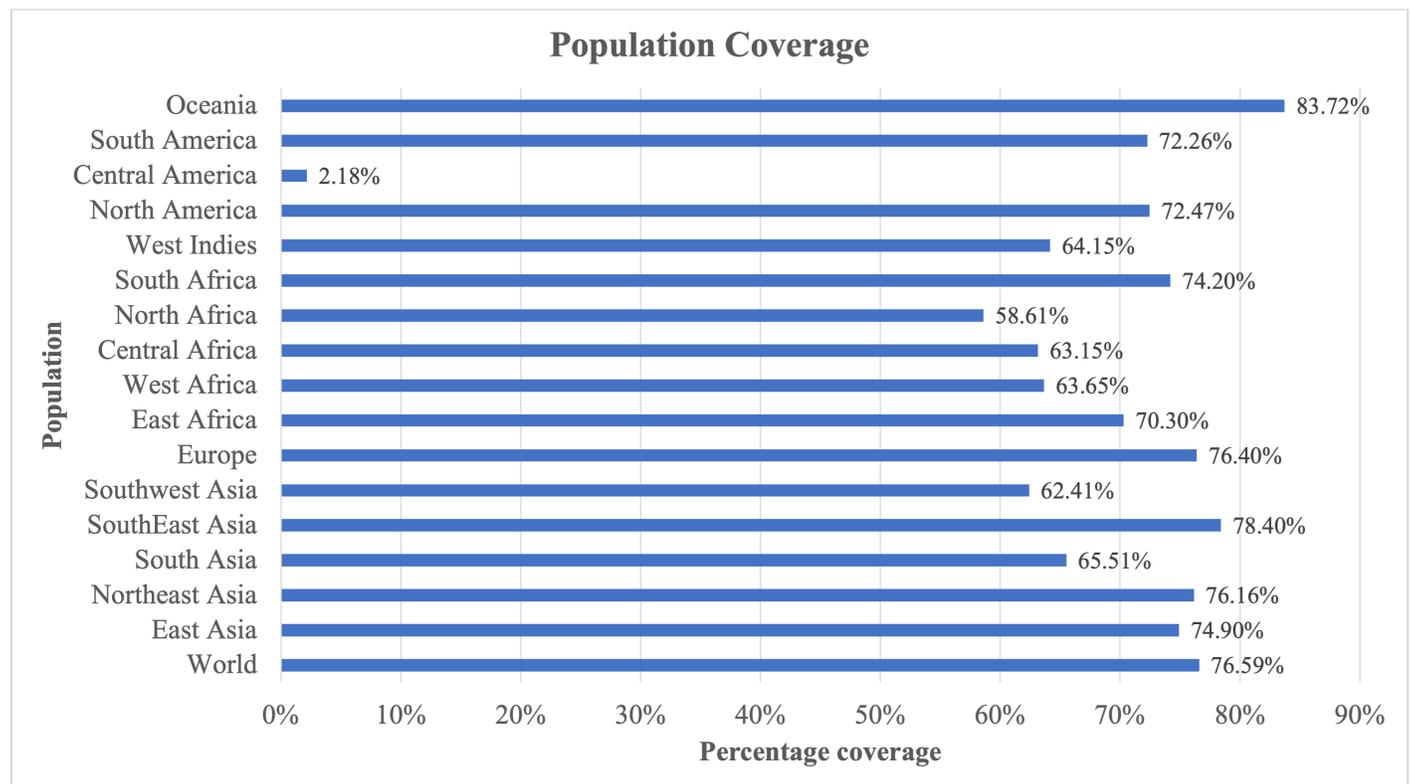


Figure 3. Population coverage of multi-epitope vaccine.

the vaccine structure predicted by First Approach Mode and Optimize Mode 0.0% with a clash score of 1.47 is much smaller than 15% of the total residue, which is around 2%–2.5%. Thus, the multipeptide structure of the vaccine produced is of good quality.

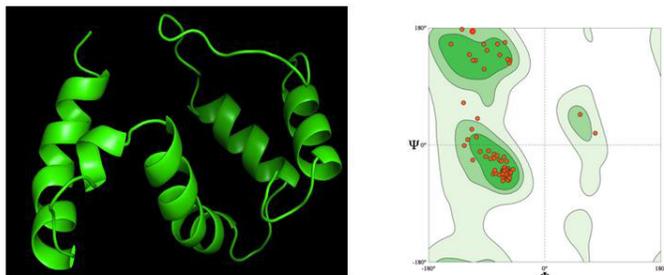
The evaluation of the tertiary structure of the vaccine was conducted by looking at the overlapped residues and Ramachandran plot analysis of the vaccine structure integrated with the DeepView (Fig. 4). Certain combinations of  $\phi$  (phi) and  $\psi$  (psi) are not allowed because they will produce a steric hindrance or an overlap between the atoms in the protein. Ramachandran plots are used to check and adjust the conformational measurements of a protein model. The Ramachandran plot has an area marked by a blue line around it, which is the coordinate area for the secondary structure of the protein. This area is the allowed region, where the number of amino acids is plotted in two regions, namely, the yellow area, namely, the area sterically permitting the values of  $\phi$  (phi) and  $\psi$  (psi), and the blue area, which is the area of maximum tolerance. steric strain. Meanwhile, the disallowed region is an area outside the allowed region, where residual plots other than glycine are not allowed in the area.

Based on Ramachandran plot analysis for 3D modeling of putative multipeptide (Fig. 4A), MolProbability score value is 1.11; Ramachandran favored value is 96.55%; Ramachandran outlier and rotamer outlier value is 0.00%; clash score value is 1.47; C beta deviation is 1 (A75 Asn); bad bonds are 0/705; and bad angels are 5/959 (Arg83, Pro84, Thr51, Asn50, Glu11, Leu43, and Ala44) (Fig. 4B).

#### Molecular docking TLR4 with putative multipeptide vaccine

Molecular docking is one of the fundamental structure-based techniques in drug design and bioinformatics, which predicts the interaction between ligand and protein or protein and protein. In immunoinformatics, docking becomes a proper tool to solve the problem of binding prediction for proteins that play a role in the immune system.

Molecular docking analysis using Rosetta web server is a multiscale Monte Carlo-based algorithm that utilized a centroid-based, coarse grain stage to identify favorable docking poses, and an all-atom refinement stage that optimizes rigid-body position and side-chain conformation. RosettaDock has been modified to overcome the critical challenge in protein-protein docking: binding-induced backbone conformational changes. RosettaDock has been applied for antibody-antigen docking research (Sivasubramanian *et al.*, 2006, 2007), peptide docking and specificity (Chaudhury and Gray, 2009; Raveh *et al.*, 2010)



**Figure 4.** (A) 3D prediction of putative multipeptide vaccine; (B) Ramachandran plot of putative multipeptide vaccine.

multi-body\_(Bocik *et al.*, 2011) and symmetric-docking (Andre *et al.*, 2008).

Generation of models ensemble in the server was conducted by 1,000 independent simulations in local docking perturbation run. Molecular docking had been run in the GrayLab. Rosetta-4 daemon and complete in 30 minutes. As shown in Figure 5, mostly all models (TLR4 with putative multipeptide) have similar conformation except model 3 (Fig. 5C), model 7 (Fig. 5G), and model 10 (Fig. 5J). From 10 models, the best model score is model 1 with a total score of  $-366.36$ , RMSD value is 18.395, the interface score (I\_Sc) value is  $-3.68$ , and I\_rmsd value is 3.049 (Table 2). The quality of docking was categorized based on the accuracy of the closest decoy in the top five scoring decoys based on CAPRI-defined criteria: high ( $I\_rmsd < 1.0 \text{ \AA}$ ), medium ( $1.0 \text{ \AA} < I\_rmsd < 2.0 \text{ \AA}$ ), and acceptable ( $2.0 \text{ \AA} < I\_rmsd < 4.0 \text{ \AA}$ ) (Méndez *et al.*, 2003). I\_rmsd or interface rmsd is calculated over all backbone atoms in interface residues with an intermolecular distance of at most 4.0  $\text{\AA}$  (Chaudhury *et al.*, 2011).

TLR4 has an active binding site as a favorable site of the multipeptide vaccine. TLR4 has two main chains such as I and J. Chain I represents Fab Hu 15C1 Heavy chain with a sequence length of 225 amino acid and no mutation. Chain J represents Fab Hu 15C1 Light chain with a sequence length of 214 amino acid and no mutation. Active binding sites favorable of multipeptide vaccine of TLR4 Chain I are S137, K134, T136, S135, P218, E217, V216, R215, G195, T196, and L194 and of Chain J are N210, R211, and G212. Amino acid residues in multipeptide that have interaction with active binding site in TLR4 are A19, L27, V28, A29, P30, F31, A32, A33, Y34, and S35 (Fig. 6).

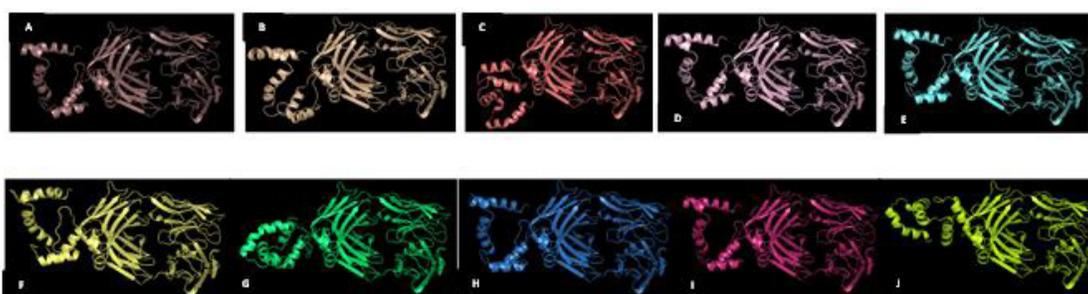
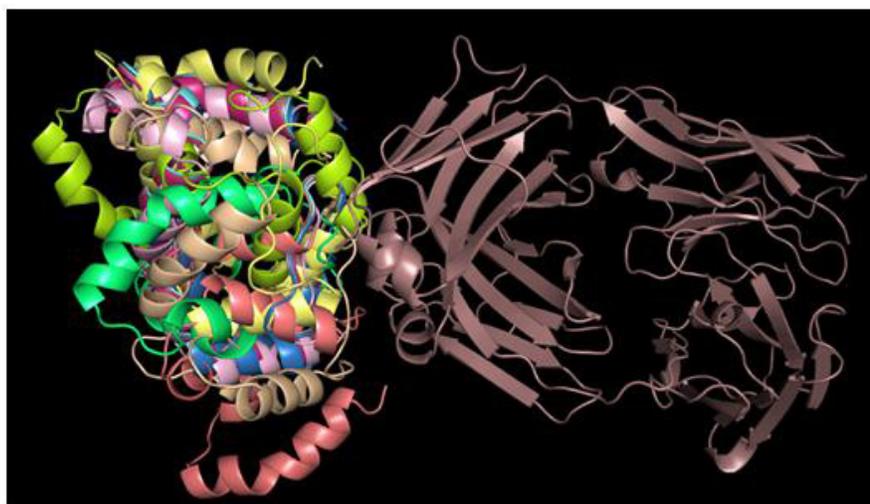
#### Molecular dynamic simulation of TLR4 and putative multipeptide vaccine

The result of the molecular dynamic simulation is diversity, consisting of RMSD, Rg, Fraction of Native Contacts (Q), RMSF, B-factor, principal component analysis (PCA), analysis of dynamic cross-correlations, analysis of hydrogen bond, and energy calculation.

Molecular dynamics (MD) simulations of single-molecule and molecular complexes, including unbiased and biased MD simulations, are widely applied to achieve accurate binding modes, binding energies of drug-receptor interactions, drug-target recognition and binding, and allosteric mechanism research. MD simulations have been successfully applied in many drug discovery researches. The information from the dynamic trajectory could be used in determining the relationship between protein structure and function.

RMSD refers to the measure of the average distance between the atoms of superimposed structures. Sometimes, equalized RMSD plots indicate the equilibrium of the system.

Based on the RMSD graph shown in Figure 7A, during molecular dynamic simulation record per frame time (ps) in X-axis, RMSD score overall is stable from 500 ps with a range of RMSD 0.0820–2.8577  $\text{\AA}$ . Based on RMSD histogram of the receptor shown in Figure 7B, RMSD value range starts from 2.0 to 2.8  $\text{\AA}$  with the highest number in value around 2.5  $\text{\AA}$ . Overall spectrum of RMSD did not show any shifts which explains structure stability and strength of vaccine inside the active site pocket.



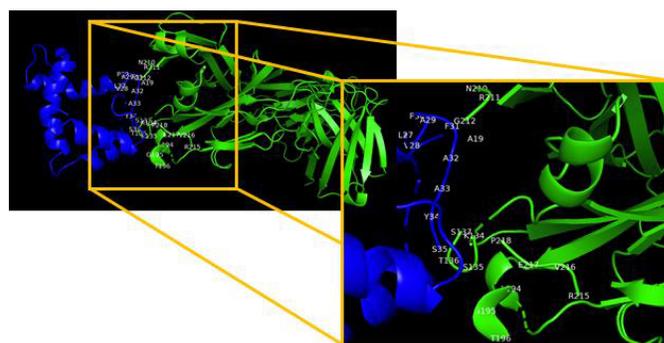
**Figure 5.** Overlapping visualization of 10 best models from molecular docking TLR4 with putative multiepitope vaccine; (A) Model 1; (B) Model 2; (C) Model 3; (D) Model 4; (E) Model 5; (F) Model 6; (G) Model 7; (H) Model 8; (I) Model 9; (J) Model 10.

**Table 2.** Docking energy of putative multiepitope vaccine with TLR4.

Model	Total score	RMSD	I_Sc	I-rmsd
1	-366.36	18.395	-3.68	3.049
2	-366.218	16.296	-3.716	6.051
3	-366.113	9.616	-3.513	6.075
4	-366.062	18.003	-3.557	5.734
5	-366.045	18.639	-3.794	5.286
6	-366.02	15.219	-3.542	5.584
7	-365.97	6.898	-3.668	4.624
8	-365.837	15.913	-3.207	5.281
9	-365.818	16.54	-3.306	4.464
10	-365.776	15.883	-3.259	5.371

Rg or gyradius is used to characterize the dynamic trajectory of flexible systems for molecular systems. The calculation of Rg is one of the important indicators used in predicting the structural activity of a macromolecule. The Rg is influenced by the changes in the folding state of the protein. This provides an important probe of the equilibrium unfolding reaction. A small Rg score indicates that throughout trajectory the proteins were in folded structure.

Based on the fraction of native contact analysis shown in Figure 8A, Q(X) value during molecular dynamic simulation per time (ps) was decreased from 0.999999165535

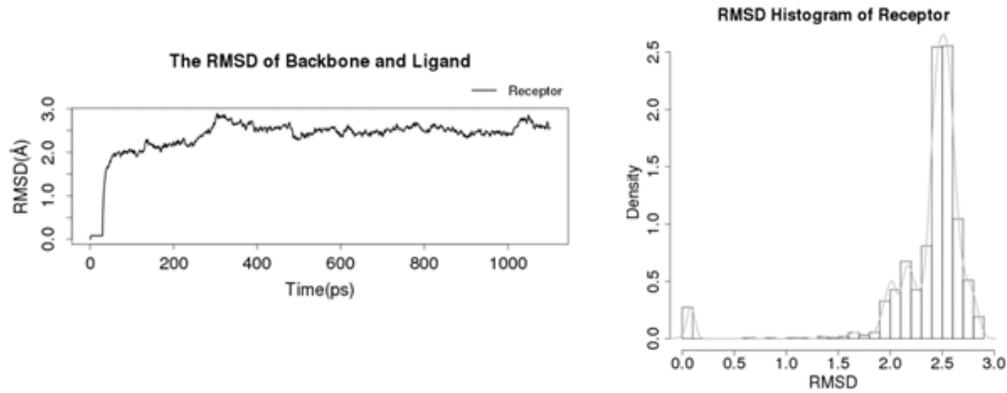


**Figure 6.** Amino acid interaction of TLR4 with putative multiepitope vaccines.

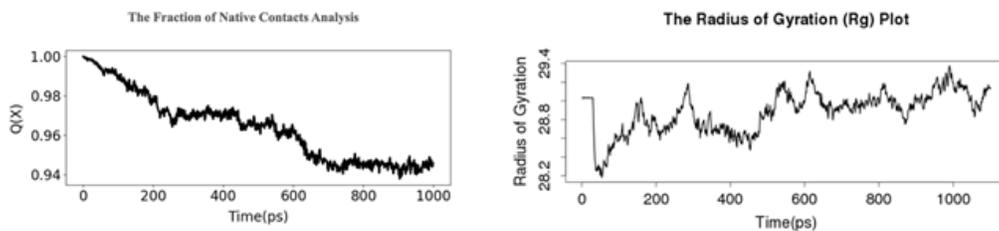
to 0.945987820625. The Rg plot in Figure 8B shows structural activity of a macromolecule during 1,000 time (ps) with a range value of 28.2–29.4.

Nonnative contacts have no significant part in the mechanism of folding in most cases. Coarse-grained theoretical and simulation models of folding support that only native contacts are energetically favorable. The fraction of Native Contacts Q(x) captured the transition sites of proteins with a folding free energy barrier. Q(x) will change with the unfolding of the protein.

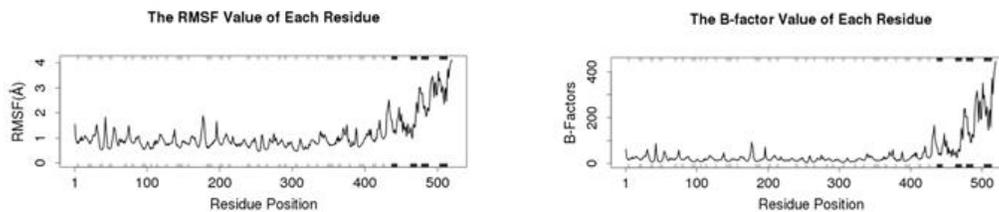
RMSF refers to the atomic positional fluctuation. Here, the fluctuation of each residue is calculated based on the CA atom of them. The residue number in the crystal structure can be got



**Figure 7.** (A) RMSD of protein backbone and ligand and (B) RMSD histogram of receptor.



**Figure 8.** (A) The fraction of native contact analysis and (B) the Rg.



**Figure 9.** (A) RMSF and (B) The B-factor.

from raw data at each residue position. At the bottom and top of the graphic, the secondary structure is indicated with colored lines (helices as black, strands as gray, and loops as white). During the simulation, these helices, strands, and loops are oriented to accommodate molecular interactions and ensure that the vaccine stays within the active site. Note that larger fluctuations are predicted for loop regions.

B-factor, or the temperature factor, is similar to RMSF, which is used to describe atomic positional fluctuations, attenuation of X-ray scattering, or coherent neutron scattering caused by thermal motion. The residue number in the crystal structure can be got from raw data at each residue position. Similar to RMSF, at the bottom and top of the graphic, the secondary structure is indicated with colored lines. Note the larger fluctuations predicted for loop regions.

RMSF value of each residue (Fig. 9A) shows a high fluctuation in residue position 430–500 and a low fluctuation in residue position 1–300. Similar to the RMSF value, the B-factor also has a low fluctuation in residue position 1–400 and has a high fluctuation in residue position 410–500 (Fig. 9B).

PCA can be utilized to analyze the relationship between different structures based on their equivalent residues and the protein trajectories. The MD trajectory analysis vignette is used to discuss the application of PCA to visualize and compare the distribution of experimental structures and MD trajectories during the simulation. Studies suggest that 3–5 dimensions are sufficient for capturing more than 70% of the total variance in MD trajectories. The eigenvalue, variance, and cumulative of the top 6 PCs can be downloaded from the raw data for PC. PCA results showed in trajectory frames which colored from blue to red in chronological order. Because there are nearly only two states for a protein, active and inactive, the frames are divided into two clusters in this server based on the top 3 PCs (Figs. 10A and B).

The atomic fluctuations of a system are correlated with one another and can be assessed by analyzing the magnitude of all pairwise cross-correlation coefficients. Blue indicates the correlated residues and red indicates the anticorrelated residues. The pairwise residues with the higher correlated coefficients ( $>0.8$ ) and with a higher anticorrelated coefficient ( $<-0.4$ ) are

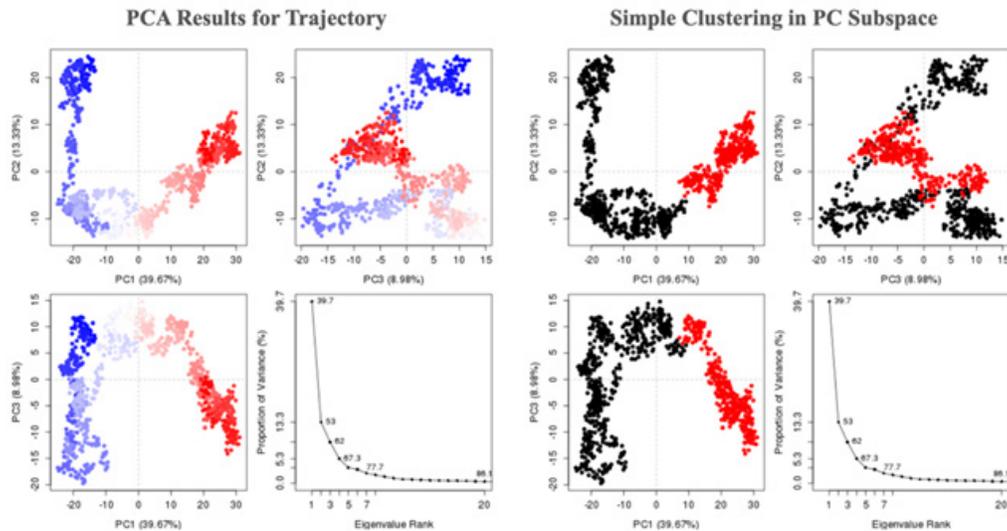


Figure 10. (A) PCA results for trajectory and (B) simple clustering in PC subspace.

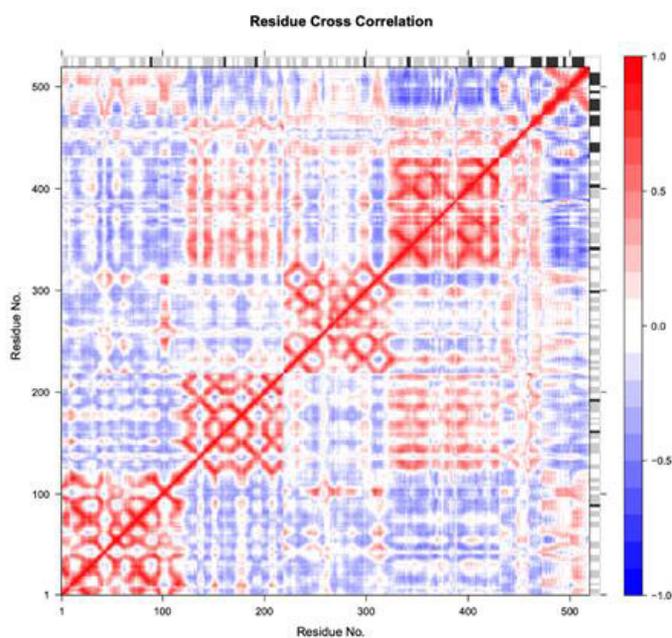


Figure 11. Residue cross correlation.

linked with light pink and light blue lines. The secondary structure schematic is added to the top and right margins of the dynamical residue cross-correlation map (helices black, strands gray, and loops white) (Fig. 11).

### CONCLUSION

In this study, we applied the immunoinformatics approach with genome diversity and conservancy analysis, *in silico* epitope prediction, molecular docking, and MD simulation in epitope-based breast cancer vaccine research. We identified seven putative epitope candidates from the conserved region of ERBB2, PTEN, and MUC4 protein, constructed as multiepitope which has the potential to be a new vaccine candidate for breast cancer. This

approach may be considered as a new, safe, and efficient approach in vaccine research. Further research studies through *in vitro* and *in vivo* tests are necessary to validate the results.

### AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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### CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

### ETHICAL APPROVALS

This is a computational study and does not involve experiments on animals or human subjects.

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