Linker Optimization in Breast Cancer Multiepitope Peptide Vaccine Design Based on Molecular Study

Fadilah Fadilah[1,2,3(✉)], Rafika Indah Paramita[1,2,3], Linda Erlina[1,2,3], Khaerunissa Anbar Istiadi[1,2], Puspita Eka Wuyung[4], and Aryo Tedjo[1,2]

[1] Department of Medical Chemistry, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
fadilah.msi@ui.ac.id
[2] Bioinformatics Core Facilities, Faculty of Medicine, Indonesian Medical Education and Research Institute IMERI, University of Indonesia, Jakarta, Indonesia
[3] Master Programme in Biomedical Sciences, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
[4] Department of Pathological Anatomy, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

Abstract. Breast cancer is most common cancer diagnosed in women. The urgency of developing effective therapeutic approaches is needed, both passive and active immunotherapy using vaccines. Immunoinformatics approach for epitope prediction of cancer proteins is one of promising approach in peptide vaccine development. Linker optimization is important parameters in peptide vaccine construction which will affect the conformation, folding and vaccine stability. From our previous study, we generate multiepitope peptide-vaccine consist of seven epitopes: DPVALVAPF, SVAYRLGTL, SQINTLNTL, RFRELVSEF, VTSANIQEF, RPRFRELVS, and MYFEFPQPL. Here we made attempt to optimize the multiepitope structure linked by 5 linker such as AAY, EAAAK, GPGPG, GGGGS, KK using in silico approach. 3D modelling of the multi epitope sequence was conducted via GalaxyTBM. Validation of tertiary structure conducted using Ramachandran plot and quality factor of the structures is being analyzed using ERRAT. Solubility of the designed vaccine was assessed using the Protein Sol webserver. The multi-epitope vaccine physicochemical parameters (pI, hydrophobicity, GRAVY, charges, and molecular weight) were conducted via Peptide Analyzing Tools from Thermofisher Scientific. From the protein validation results and physicochemical features, the best peptide model is model 1 which linked with EAAAK linker. Model 1 can be used as potential multi-epitope agents for breast cancer vaccines.

Keywords: Breast cancer · Linker · Immunoinformatics · Multi-epitope · Vaccine

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1 Introduction

Breast cancer is most common cancer diagnosed in women and number of cases increasing and expected to continue to rise [1]. The high incidence made urgent research for effective therapies for cancer [2]. Limitations of passive therapy made active immunotherapy as potential approach in cancer therapy. Active immunotherapy using vaccine will generate prolonged activation of the immune system [3].

One of promising vaccine development methods is by using immunoinformatics approaches. By using genomic data, this reverse vaccinology approach will predict epitopes which interact with human leukocyte antigen (HLA) and have an important role in immunity [4]. Predicted epitopes from the in silico approach can be processed to in vivo analysis and accelerate the vaccine development [5].

Proteins which are overexpressed in cancer patients is subjects of active immunotherapy. In our previous study [6], potential epitopes as breast cancer vaccines have been predicted using ERBB2, MUC4, and PTEN proteins. These potential epitopes are designed from the conserved region of the proteins to address the diversity of proteins due to mutation in cancer patients [7].

Potential epitopes generated from reverse vaccinology approach might be constructed into a multiepitope construct, linked by linkers and addition of adjuvant. Compared to single-epitope vaccines, multiepitope vaccines are more cost-effective, time-saving, stable, and specific. By combining several potential epitopes, the multiepitope vaccine might induce immunity against multiple antigenic targets [8]. One of the important strategies in multi-epitope construction and peptide fusion is the choice linker.

Linkers or spacers are short amino acid sequences which separate domains or peptides in a protein. Linker generally classified into three categories: the flexible, rigid, and in vivo cleavable linkers [9]. Most linkers have rigid properties which prohibit unwanted interactions between domains [10]. Flexibility or rigidity of the linker is an important parameter that affects the function of proteins [11]. Different linkers may result in different structure and also affect biological activities and expression.

Linker optimization is necessary in designing effective peptide vaccines [12]. Linkers play roles in flexibility, folding activities and separation of functional domains [13]. Moreover, linker utilization in peptide-vaccine design aims to minimize the junctional immunogenicity and facilitate immune processing of vaccines in the cells and ensuring the immunogenicity of epitopes. The choice of linkers used in protein was based on the stability of 3D structure. Bioinformatics approach is one of promising tools in molecular studies between the structure and activity of multi epitope and fusion protein [14]. Linker optimization in peptide-vaccine design is conducted in this research based on molecular approach with focus on structure analysis.

2 Methods

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

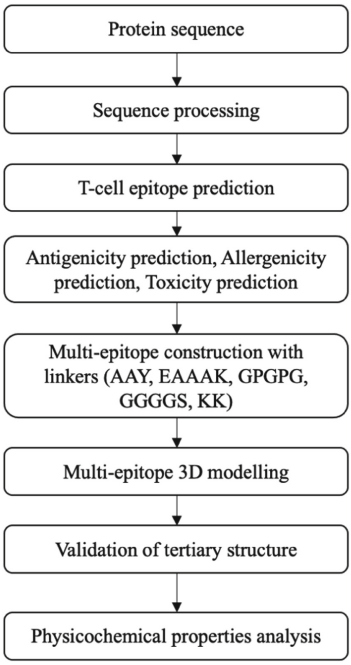

Fig. 1. The methodology of the research.

2.1 Data Retrieval, Sequence Analysis and Epitope Predictions

ERBB2, PTEN, and MUC4 protein sequences were downloaded from the UniProt web server (https://www.uniprot.org/) with accession number P04626, P60484, Q99102 respectively [15]. Missense mutation data of the protein were retrieved from COSMIC database (https://cancer.sanger.ac.uk/cosmic). Conserved regions of the sequence were determined by sequence entropy using AVANA software [16]. T-cell epitopes were predicted from the conserved peptide using NetCTL 1.2 (https://services.healthtech.dtu.dk/service.php?NetCTL-1.2) [17]. Immunogenicity characteristics of epitopes were predicted using IEDB Immunogenicity webserver (http://tools.iedb.org/immunogenicity/), antigenicity characteristics via VaxiJen 2.0 page (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), allergenic characteristics on the AllerTOP page (https://www.ddg-pharmfac.net/AllerTOP/), and the toxicity ToxinPred page (http://www.imtech.res.in/raghava/toxinpred/) [18]–[21].

2.2 Multiepitope Construction

Each of epitopes from the previous study was then linked with several linkers: AAY, EAAAK, GPGPG, GGGGS, KK to construct a putative multiepitope. The HEYGAEALERAG motif was added and linked by EAAAK to the epitopes to enhance epitope presentation [6].

2.3 Multiepitope Modelling

3D modelling of the multi epitope was conducted via GalaxyTBM (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=TBM) based on template-based modelling [22]. GalaxyTBM produce core structures using HHsearch scoring, alignment using PROMALS3D and the ULR modelling using optimization modules in GALAXY [23–25].

2.4 Validation of Tertiary Structure

Validation of tertiary structure conducted using Ramachandran plot using Zlab webserver https://zlab.umassmed.edu/bu/rama/ [26]. All parameters are observed such as total amino acid, preferred conformation, and questionable conformation. All glycines, prolines and display labels for outliers were chosen for Ramachandran plot visualization. Quality factor of the structures is being analyzed using ERRAT in SAVES v.6.0 server (https://saves.mbi.ucla.edu/) [27].

2.5 Physicochemical Properties Analysis

Solubility analysis of the designed vaccine conducted on Protein Sol webserver (https://protein-sol.manchester.ac.uk/) [28]. Other physicochemical parameters analysis were conducted via Peptide Analyzing Tools from Thermofisher Scientific (https://www.thermofisher.com/id/en/home/life-science/protein-biology/peptides-proteins/custom-peptidesynthesis-services/peptide-analyzing-tool.html). These physicochemical features being analyzed are pI, hydrophobicity, GRAVY, charges, and molecular weight.

3 Results and Discussion

3.1 Prediction of T-cell Epitopes

Epitope, an antigenic determinant, is part of antigen recognized by the B cells and T cells antibodies. Previous study [6] showed that from the CTL prediction tools, DPVALVAPF, SVAYRLGTL, SQINTLNTL, RFRELVSEF, VTSANIQEF, RPRFRELVS, and MYFEFPQPL was selected as potential peptides in multi-epitope vaccine.

3.2 Multiepitope Construction and Modelling

Predicted T-cell epitopes were constructed as a multi-epitope model link with several linker: EAAAK, GPGPG, GGGS, KK, and AAY, as shown at Fig. 2. EAAAK is a rigid alpha-helix peptide linker and offer efficient separation of functional domains thus maintain epitopes functional properties [29]. The GPGPG linker was designed and demonstrated to be able to induce immunity [30]. GGGS is one of the flexible linkers widely used in fusion proteins. KK (bi-lysine) linker is the target sequence of lysozomal protease in antigen processing and are primarily associated with the independent immunoactivities of a vaccine [31]. AAY, known as proteosomal cleavage sites in mammalian cells, also used as linker in this research to increase stability of proteins [32].

3D structure modelling was conducted based on template-based modelling in Galaxy TBM server. The 3D structure of multi-epitope with each linker is shown at Fig. 3, with red coil representing alpha-helix and yellow sheet representing beta-sheet.

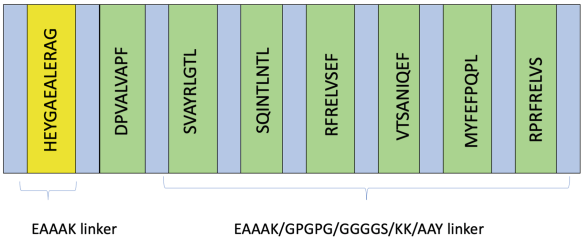

Fig. 2. Multi-epitope sequence retrieved from previous study and linkers used for the model construction in this study.

Table 1. Validation from Ramachandran plot and quality score ERRAT results

Model	Total amino acid	Preferred conformation	Questionable conformation	ERRAT Quality Score
1	110	106	4	97.21%
2	76	76	0	93.617%
3	82	76	6	82.278%
4	89	85	4	88.506%
5	96	91	5	85.106%

3.3 Tertiary Structure Validation

Validation of tertiary structure was conducted using the Ramachandran plot, as shown in Fig. 4 and the values are shown in Table 1. Ramachandran plots are used to measure the conformation of the model protein by looking at the overlapping residues. Combinations of phi/psi are not allowed due to high probability in steric hindrance generation. Based on Ramachandran plot results on Table 1, all models have more than 90% preferred conformations and all models had more than 90% of the amino acid in the allowed region hence verify the structural stereochemical quality. Conformational analysis of Ramachandran plot can be used to determine whether the obtained backbone conformation is generated correctly. Identification of specific interactions affecting the backbone of glycine and pre-proline can be used in for structural motifs analysis containing these residues.

ERRAT server used for statistical analysis of non-bond interaction between different types of atoms. ERRAT is used for verify protein structured determined by crystallography [27]. Results from ERRAT analysis shown by the Fig. 5. Yellow region is region of structure that can be rejected at the 95% confidence level; 5% of a good protein structure is expected to have an error value above this level. Red regions are regions that can be rejected at 99%.

The server calculated a quality score of models 1 value 97.21%; model 2 value 93.617%; model 3 value 82.278%; model 4 value 88.506% and model 5 value 85.106% for the vaccine. The best model is model 1 with the highest value 97.21% (Fig. 5).

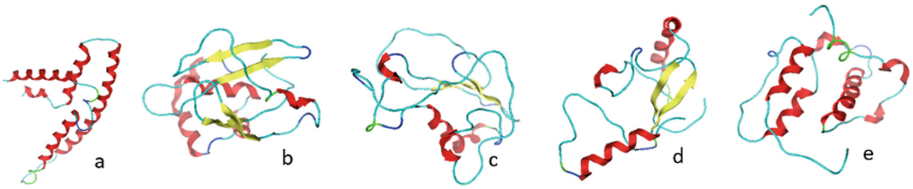

Fig. 3. 3D prediction of multi-epitope, (a) model 1 with EAAAK linker, (b) model 2 with GPGPG linker, (c) model 3 with GGGGS linker, (d) model 4 with KK linker, (e) model 5 with AAY linker.

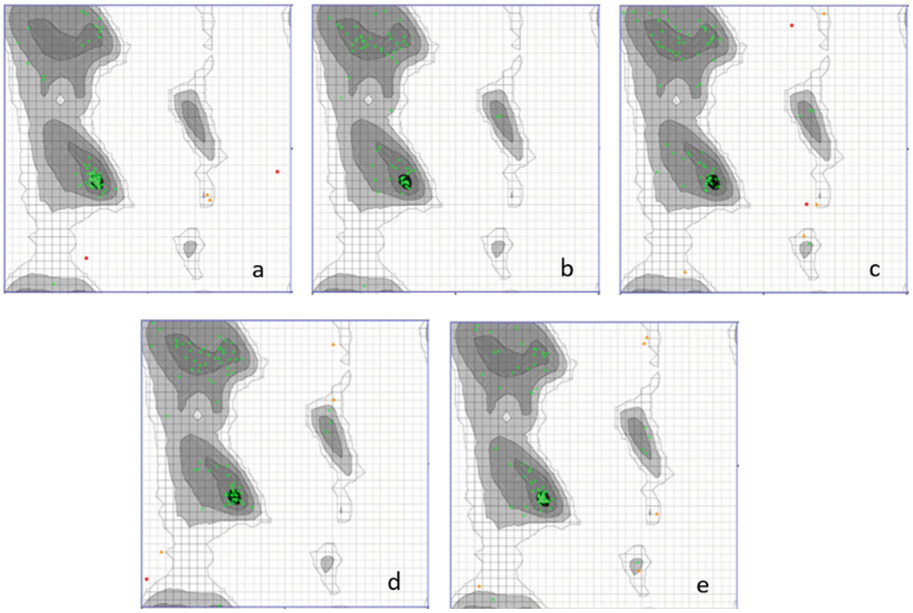

Fig. 4. The Ramachandran plot validation of multi epitope models with different linkers, (a) EAAAK linker, (b) GPGPG linker, (c) GGGGS linker, (d) KK linker, (e) AAY linker.

3.4 Physicochemical Properties of Multi Epitope Sequence

The physicochemical properties and characterization of the amino acids can be predicted using computational tools used in proteomics and molecular biology. This amino acid sequence information can be used to identify functional targets.

Epitope models 1–3 have sequence length 120 aa, model 4 has sequence length 99 aa and model 5 has sequence length 106 aa. Molecular weight of all models ranges from 11343.38–12842.67 KDa. As shown in Table 2, all structures showed different GRAVY values, measuring the hydrophobicity or hydrophilicity of the structures. The GRAVY value calculated from the sum of hydropathy values of all amino acids then divided by the number of residues in the sequence [33]. The range of GRAVY values are −2 to +2; negative score means hydrophilicity and positive score indicates hydrophobicity [34]. Proteins with a more negative GRAVY score considered hydrophilic with good

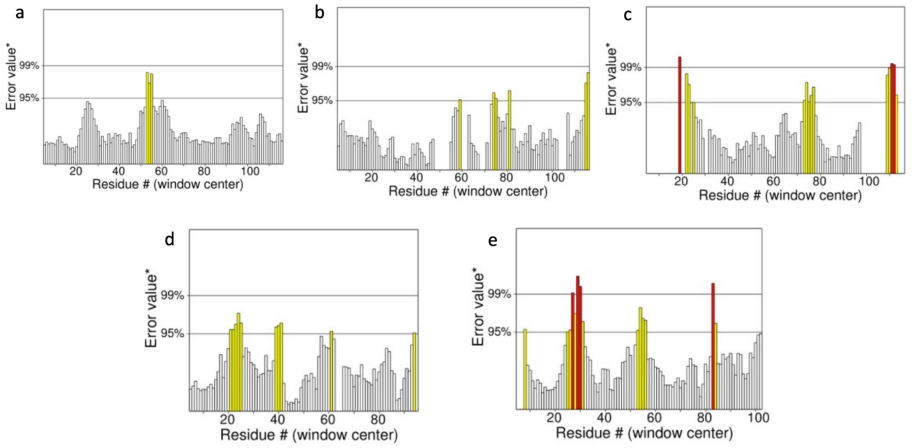

Fig. 5. Structure validation of multi-epitope vaccine model structure using ERRAT: (a) Model 1, (b) Model 2, (c) Model 3, (d) Model 4 and (e) Model 5.

Table 2. Physicochemical properties of design multi epitope

Model	Sequence length	Hydrophobicity	GRAVY	MW	PI	Solubility
1	120	62.73	−0.19	12842.67	5.4	0.672
2	120	52.01	−0.33	12099.12	5.2	0.492
3	120	48.02	−0.21	11748.76	5.2	0.458
4	99	52,76	−0.64	11343.38	10.6	0.75
5	106	76.82	0.07	11678.89	5.2	0.441

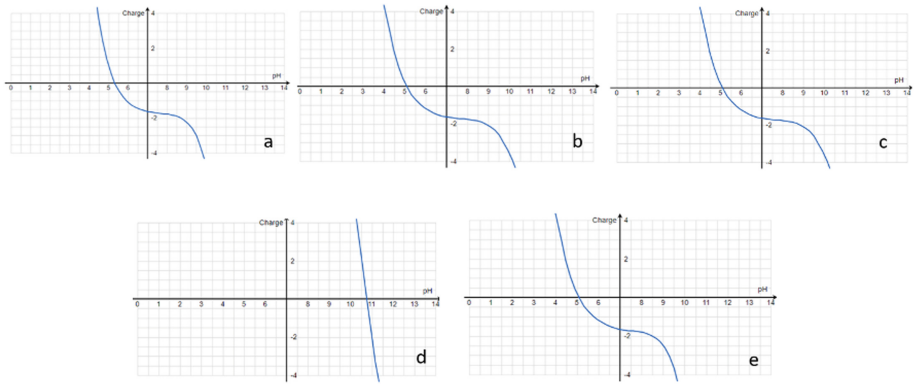

Fig. 6. The charge of a multi epitope model with different linkers, (a) EAAAK linker, (b) GPGPG linker, (c) GGGGS linker, (d) KK linker, (e) AAY linker.

solubility which show strong interaction with water molecules [35]. GRAVY values for model 1, model 2, model 3 and model 4 were negative, indicating slightly hydrophilic properties.

Their theoretical pI values were also very close, except for model 4, which theoretical pI was calculated as 10.6. pI is isoelectrical point at which the total liquid charge of the amino acid or protein molecule is zero [36].

Protein solubility prediction based on physicochemical properties is important in therapeutic applications. The protein charge analysis showed that the majority of models tend to have negative charge properties. The negative charge correlates strongly with protein solubility. Protein solubility prediction on the ProteinSol server is given in the 0–1 range, more closely to 1, more soluble the protein in water. From the Table 2 above, solubility of model 4 is the highest with the value 0.750 and solubility of model 5 is the lowest with the value 0.441. Model 4 and 1 tend to be more soluble in water than the other models. This indicates that this model is highly soluble upon expression in the vector and not forming an insoluble aggregate. High solubility protein will provide advantages during production of the protein [37, 38].

The advancement of immunoinformatics tools in the post-genomic era enables explorations of several platform in developing high immunogenic multi-epitope vaccine. The physicochemical analysis, solubility and other molecular prediction confirm the potential of the vaccine for further evaluation of experimental immune response [39].

The multiepitope peptide-vaccine consist of seven epitopes linked by EAAAK linker showed the best in validation score and physicochemical properties. EAAAK linker is a rigid alpha-helix peptide linker and offers efficient separation of functional domains thus maintaining epitopes functional properties play roles in flexibility, folding activities and separation of functional domains. This model can be used in development and design of multi epitope agents of breast cancer vaccines.

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Author Contribution. FF, RIP, LE, KAI, PEW, AT 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 authors read and approved the final manuscript.

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