RESEARCH ARTICLE

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# Reverse Vaccinology and Immunoinformatics Strategy to Screen Oncogenic Proteins and Development of a Multiepitope Peptide Vaccine Targeting Protein Kinases against Oral Cancer: An *in-silico* Study

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## Abstract

**Background:** Despite standard therapies and immunotherapies, the mortality rate of patients with oral cancer remains high. Therefore, there is a need for more effective and targeted treatments. Multi-epitope vaccines have been developed for various cancers owing to their easy protection and delivery. However, no multi-epitope vaccine has been designed to prevent oral cancer. **Methods:** In this study, a reverse vaccinology approach, along with various machine-learning integrated immunoinformatics tools, was used to design a multi-epitope peptide vaccine. **Results:** Using an integrated computational method, *LYN* Proto-Oncogene and *AKT1* were identified as good candidates. Both *LYN* and *AKT1* are protein kinases and plays a central role in regulating various outputs, such as proliferation, differentiation, apoptosis, and migration in cancer. These proteins were selected because of their favorable physicochemical properties, non-allergic, non-toxic, and antigenic nature. Suitable B and T cell epitopes were identified based on their physicochemical characteristics, toxicity, allergenicity, antigenicity, and immunogenicity. A vaccine was constructed using these immune epitopes and *TLR4* agonist as an adjuvant. Molecular dynamics simulation suggests strong binding affinity for Toll-like receptor 4. Furthermore, immune simulation studies suggest the activation of immune cells and a strong IgG/IgM response for approximately one year. **Conclusion:** We propose that the vaccine developed has high immunogenic potential and able to induce both cell mediated and humoral immunity against oral cancer.

**Keywords:** Oral cancer- Reverse vaccinology- multi-peptide vaccine- Molecular dynamics simulation

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## Introduction

Oral squamous cell carcinoma that arises from the oral cavity and lips is a major international health problem, with an annual incidence of 389,846 and 188,438 deaths, respectively, according to data from GLOBOCAN 2022. Oral cancer, or cancer of the mouth, is the 16th most prevalent cancer worldwide, with the highest number recorded in Asia (GLOBOCAN 2022). Nevertheless, the number of young patients has increased dramatically, especially those with tongue cancer, in the last 10 years. Reportedly, less than 50% of oral cancer patients survive after a confirmed diagnosis [1]. Therefore, more effective and targeted treatments are required for this life-threatening disease.

Oral cancer is treated with surgery, chemotherapy, and radiotherapy. Immunotherapy in the field of oral cancer treatment may be the fourth pillar that supports surgery, chemotherapy, and radiotherapy [2]. For instance, immunostimulants can enhance the production of tumor-specific antibodies, cytokines, and immune cells. The activation of immune cells ultimately aids in the destruction of cancer cells. For example, CD8+ T cells are activated by IL-12, which is released from antigen-presenting cells to kill tumors. Similarly, helper T lymphocytes (Th1 and Th2) play an essential role in the destruction of tumor cells. Th1 cells produce interferon gamma (IFN-γ), which is released from Th1 cells and activates CD8+ T cells, whereas IL-4 is released from Th2 cells and plays an essential role in activating B cells, thereby inducing an antibody-mediated immune response. Therefore, peptide-based vaccines have attracted great interest because they can stimulate both humoral and cell-mediated immune responses [3]. Moreover, these vaccines are considered appropriate because of their ease of protection and delivery to the gut using various strategies.

Peptide vaccines mimic known or predicted

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tumor epitopes that can be either tumor-specific or tumor-associated antigens. There are two types of peptide-based vaccines: i) single-epitope peptide vaccines and ii) multi-epitope peptide vaccines. Multiple epitopes allow broader coverage of HLA and have several advantages over conventional single-epitope vaccines: i) they are made up of several epitopes, such as cytotoxic T lymphocytes (CTL), Th, and B cell epitopes, which can stimulate both T- and B-cell-mediated immune responses; ii) if multiple epitopes are from different tumors, this approach can be used for various tumors; iii) epitopes when linked with adjuvants can improve long-term immune responses; and iv) multi-epitope vaccines are usually longer and therefore presented by MHC molecules after processing. After internalization, some parts of the longer peptides are degraded via the endosomal pathway, which activates CD4+ T cells, whereas other parts enter the cytoplasmic pathway and activate CD8+ T cells. v) More specific anti-tumor response [4]. The reverse vaccinology approach uses genomic information derived from in silico analysis of an organism to develop a multi-epitope peptide vaccine. This approach speeds up the search for novel vaccine candidates in comparison with the conventional vaccinology approach [5].

In this study, we used a reverse vaccinology approach and machine-learning-based immunoinformatics tools to identify suitable vaccine candidates for oral cancer. The *AKT1* and *LYN* were identified as promising candidates for immunization. Both of these proteins play a central role in tumorigenesis including oral cancer [6-9]. The final vaccine contained multiple B- and T-cell epitopes from these two proteins, along with a TLR4 agonist as an adjuvant that can activate both humoral and cell-mediated immunity. We also identified a strong interaction between the vaccine and the TLR4 receptor, suggesting its immunogenic nature.

## Materials and Methods

*Data acquisition*

To identify a suitable vaccine candidate, data were acquired using recent and relevant extensive literature searches and clinical trial databases of clinicaltrials.gov, a publicly available resource. In the literature search, the following words were entered on the NCBI, Medline and Embase such as oral cancer, vaccine, cancer, immunotherapy in cancer, cancer vaccines, clinical trials on cancer vaccines, and drugs. The search parameters on clinicaltrials.gov included the following parameters: 1). Study phase: Early phase 1, phase 1, phase 2, and phase 3; and 2). Study type: Interventional; 4) Sex: All. Studies that were terminated, suspended, or withdrawn during clinical trials were excluded. Studies that did not have full-text availability, duplication of genes, and no relevance of titles and abstracts were excluded. Studies were also excluded for the following reasons: not presenting statistically significant data and not focusing on cancers, cancer vaccines, cancer drugs, or chemotherapy.

By applying these search criteria, we identified 54 relevant genes after screening 2179 clinical trials and 251 research papers for the development of vaccines against oral cancer. Reverse vaccinology approach was used to identify potential vaccine candidates and development of vaccine (Figure 1). The genes were shortlisted on the basis of their significance on immunotherapeutic responses and tumor suppressive properties.

*Ethical approval*

Ten patients with technically unresectable oral cancer (referred to 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/00/081734/5307). The study was conducted in accordance with the Declaration of Helsinki. The patients provided written informed consent to participate in an institutional review board-approved research protocol. All methods were performed in accordance with the relevant guidelines and regulations provided by the Ethics Committee of Parul University. Patients who had undergone prior chemotherapy and those with known additional malignancies that progressed or required active treatment in the past 2 years or had salivary gland disease were excluded from the study. All patients were treated with two cycles of docetaxel, cisplatin, and 5FU for 42 days (21 days/cycle).

*Identification of surface proteins*

Proteins present on the surface are considered ideal candidates for vaccine development because of their interaction with immune cells and antigen-presenting cells (APCs). Using the VaxElan server, we tested all 54 genes to determine their localization. The VaxElan server defines the cellular localization of proteins based on a score of 0 and 1, where 0 and 1 indicate cytoplasmic and surface localization, respectively. A value between 0 and 1 suggests that the location of the protein is uncertain. Only those proteins were chosen for vaccine development, which had a value of one on the VaxElan server [10].

*Trans-membrane (TM) analysis*

Proteins with more than one transmembrane domain are considered difficult to express and have difficulties in their purification processes. Therefore, these proteins are not considered to be suitable for vaccine design. Two online servers, HMMTOP [11] and TMHMM [12], were used to identify TM domains, and proteins with 0 or 1 transmembrane domains in their structures were chosen for vaccine design. These servers predict the transmembrane helices. TMHMM can also differentiate between soluble and membrane proteins with greater than 99% specificity and sensitivity [12].

*Instability index analysis*

The ProtParam tool and 'peptides' package of RStudio were used to screen 54 antigenic proteins on the basis of their instability index, with default values for all parameters. Proteins with an instability index of < 40 were chosen for vaccine design [13].

*Antigenicity prediction*

The antigenicity of all 54 proteins was predicted using

the VaxiJen v2.0 server as described previously by our group [14, 5]. Only proteins with antigenicity values greater than 0.5 were chosen for further analysis.

*Allergenicity prediction*

The AllergenOnline database and AllerTOP v. 2.0 servers were used to identify allergenicity of the proteins, as described previously by our group [15, 5].

*Evaluation of filtered proteins*

The physiochemical characteristics of filtered protein were estimated using 'peptides' package of RStudio and ProtParam. These tools define ten structural properties of proteins, as described previously by our group [16, 5]. The GRAVY value of a protein was calculated using the length of the query sequence and the hydropathy value of each amino acid. Positive and negative GRAVY scores define hydrophobic and hydrophilic natures, respectively.

*B-cell epitope recognition*

B cell epitopes induce large amounts of antigen-specific antibodies and are, therefore, considered good vaccine candidates. The B-cell epitope in the filtered protein (*LYN* and *AKT1*) was predicted using the ABCPred server with default settings. The server predicted B-cell epitopes with 15 amino acid residues [17]. B-cell epitopes were also evaluated for IFN-γ and IL-10 using the IFNepitope and IL10Pred servers. IFNepitope server predict IFN-γ inducing peptides compared to other cytokines [18]. The IL-10Pred server predict IL-10 inducing peptides using a random forest probability approach [19].

*T-cell Epitope Prediction*

The Immune Epitope Database (IEDB) was used to identify CTL epitopes having nine amino acids using the stabilised matrix method (SMM) and NetMHCPan 4.1 EL (Supplementary Table S1) (http://tools.iedb.org/mhci/). The most frequently occurring MHC alleles were used as a reference set, using the default settings of the IEDB server. The NetMHCPan 4.1 EL server used a percentage score to discriminate between strong (rank < 0.5%) and weak MHC binders (rank < 2%). Helper T lymphocyte (HTL) epitopes with 15 amino acids were also predicted using the stabilized matrix method (SMM) and NetMHCIIpan-4.1 EL (Supplementary Table S2). Fifteen human HLAs were used as the reference sets in the prediction process. The SMM server predicted the binding affinity of epitopes with MHC based on IC50 values (high affinity: IC50 ≤ 50 nM, moderate affinity: IC50 ≤ 500 nM, and poor affinity: IC50 ≥ 5000 nM) [20] and NetMHCIIpan-4.1 server predicted binding affinity based on percentile rank (strong binders rank < 2% and weak MHC binders rank < 10%) [21].

*Antigenicity, allergenicity, toxicity and immunogenicity of the immune epitopes*

Epitopes with antigenic scores of more than 0.5 were evaluated for their allergic and toxic nature. Allergenicity was assessed using the same servers as described above. The ToxinPred server was used to predict toxicity [22].

*Real time polymerase chain reaction*

RNA extraction from 10 oral cancer patients was performed using the Trizol (Qiagen, Cat # 79306) method, as described previously [23]. cDNA was synthesized from 500 ng of RNA using a G-Biosciences cDNA Synthesis Kit (Cat # 786-5020). The resulting cDNA was used for qRT-PCR (Rotor-Gene Q; Qiagen). qRT-PCR was performed in triplicates with 2X SYBR Green qPCR Master Mix from G-Biosciences (Cat # 786-5062) 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 TCCCTGAGGCCATTTCTTTCATAGATG, human *AKT1* reverse GCAGCTGACCCATGAAGTTGAAGT, human *LYN* forward AGTCTCTATGGCAGACCTTAGACC, human *LYN* reverse TTTCTGGAGATTCTTTCTGTAGCC, human beta actin forward GGACTTCGAGCAAGAGATGG, and human beta actin reverse AGCACTGTGTTGGCGTACAG.

*Protein expression analysis*

The subcellular localization and expression of *AKT1* and *LYN* 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 [24].

*Construction of the multi-epitope vaccine candidate*

Adjuvants are necessary to enhance the immune response during subunit vaccination. Therefore, 50S ribosomal protein L7/L12 (accession no: P9WHE3.1), a TLR4 agonist, was selected as an adjuvant to boost vaccination immunogenicity. The therapeutic potential of synthetic TLR4 agonists has been demonstrated in several studies. For example, in clinical trials, monophosphoryl lipid A (MPL) has been used as a therapeutic vaccine against cancer [25]. Vaccine was constructed using linkers and adjuvants as described previously by our group [5]. Antigenicity, allergenicity, toxicity, and physicochemical characteristics were evaluated as described above.

*3D structure refinement and validation*

The I-TASSER server was used to estimate a three-dimensional model of the vaccine construct. It uses LOMETS, a multithreaded technique, to identify templates [26]. The 3D structure was further refined using GalaxyRefine server. The stereochemical properties and accuracy of the refined structure were evaluated using a Ramachandran plot, ERRAT (https://saves.mbi.ucla.edu/), and the ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) server (last accessed date: 03-01-2024) [27]. Antigenicity, allergenicity, and toxic properties of the refined construct were determined as previously described.

*Molecular docking of vaccine-TLR4 complex*

The ClusPro server was used to dock the toll-like receptor (TLR4-PDB ID:4G8A) and vaccine construct [28]. The UCSF chimera was used to assign charges,

add hydrogen atoms, and remove water molecules from both the vaccine (ligand) and TLR4 (protein) [29]. The HDOCK server was used to predict the binding affinity of the peptide-protein complexes [30].

*Molecular dynamics simulation*

GROMACS version 2022.5 was used to simulate the interaction between TLR4 and multi-epitope vaccination design [31]. A CHARMM force field is applied to the system. The built-in commands of the system were used for solvation, ion addition, and energy minimization. The simulation was conducted 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) were calculated. After the 150 ns MD simulation, the binding free energy (ΔG) was calculated using the NAMDenergy plugin in NAMD [32].

*Characterisation of immune responses of the vaccine construct*

The C-immSim immune server with default parameters was used to assess the immunological responses of the refined vaccine construct [33]. This service uses a position-specific scoring framework (PSSM) to simulate and predict immunological interactions with immunogenic epitopes. The server was run at default parameters, with antigenic injections given at four-week intervals.

*Codon adaptation of vaccine construct*

The resulting amino acid sequences were translated into their corresponding nucleotide sequences [34]. The Java Codon Adaptation Tool (JCat server) was used to optimize codons in the E. coli strain K12 [35]. Finally, using Snap Gene v4.2 software (https://snapg ene.com/), the modified nucleotide sequence was introduced into the pGL4.10[luc2] expression vector.

## Results

The most promising vaccine candidate against oral cancer was identified in the current investigation using computational methods and reverse vaccinology.

*Reverse vaccinology pipeline to find a suitable vaccine candidate*

The reverse vaccinology pipeline shortlisted two out of 54 genes as suitable vaccine candidates against oral cancer: 1) *LYN* Proto-Oncogene from Src Family

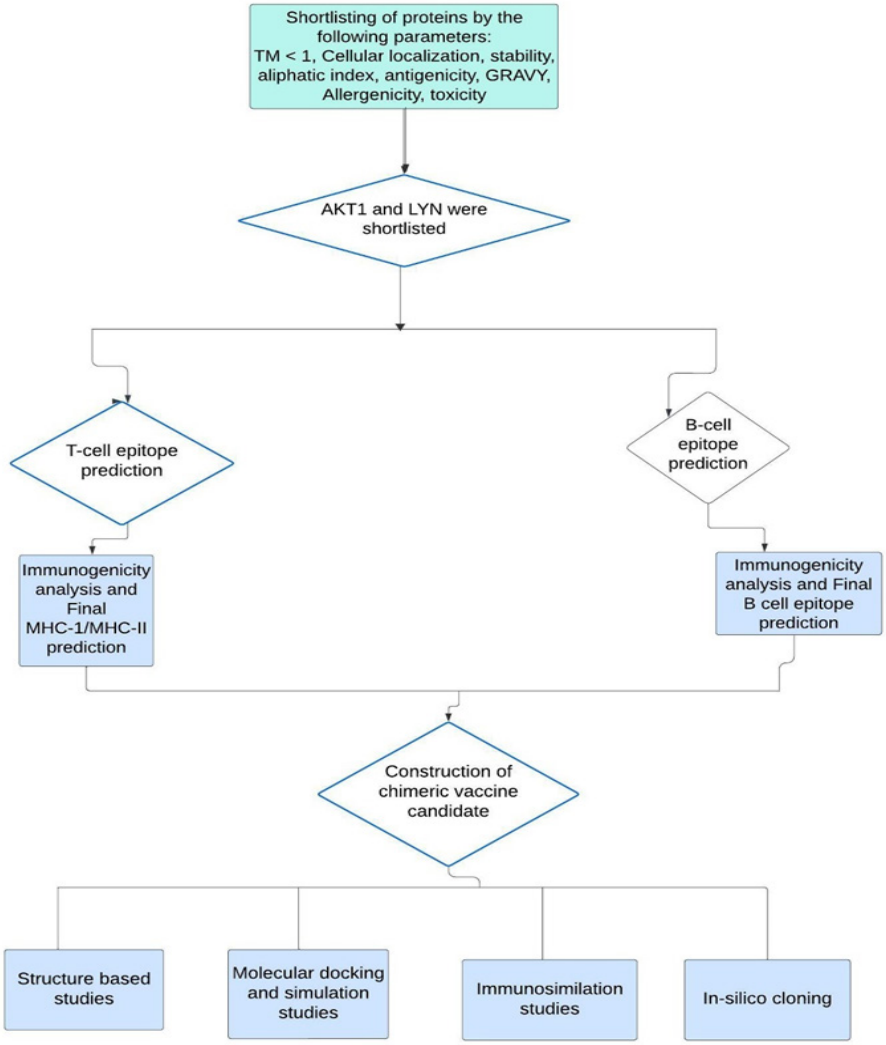


Figure 1. Flow Chart to Identify Potential Vaccine Candidates Using Reverse Vaccinology Approach and Development of Vaccine Candidate

Table 1. B-cell epitopes present on surfaces of *LYN & AKT1*

| Protein | Antigenic B-cell epitopes | Antigenic score | Allergenicity | Toxicity | IFN Server | IL10 inducer |
|---|---|---|---|---|---|---|
| *LYN* | FKDITRKDAERQLLAP | 0.8 | N | N | Positive/0.489 | Positive/0.423 |
| *AKT1* | KEKATGRYYAMKILKK | 0.5 | N | N | Positive/1.074 | Positive/1.332 |

Tyrosine Kinase and 2) *AKT1* (AKT Serine/Threonine Kinase 1). These proteins are shortlisted based on the following characteristics: cellular localization, number of transmembrane domains, allergenicity, toxicity, GRAVY, and pi values. Both of these proteins have no transmembrane domain (TM=0), non-allergic, and non-toxic. Using the VaxElan predictions and algorithm, the calculated Pi score was 0.7, indicating that both *LYN* and *AKT1* genes have a 70% chance of being suitable vaccination candidate for Oral Cancer (Supplementary Table 1).

The physicochemical attributes of these proteins were also evaluated. A low instability index and high aliphatic index suggest protein stability. Additionally, both genes had negative GRAVY values, suggesting their hydrophilic nature (Supplementary Table 2).

*Identification of suitable B-cell epitopes in LYN and AKT1*

B-cell epitope vaccines induce a natural humoral immune response and therefore have high relevance [36]. The ABCPred server predicted 98 B-cell epitopes in *LYN* and 28 epitopes in *AKT1*. Of these 98 epitopes, two epitopes, one for each protein, were shortlisted based on their antigenic score, allergenicity, and toxicity. These epitopes are antigenic, non-allergenic, and nontoxic (Table 1).

These epitopes have also been shown to induce IL-10 and IFN production. IFN-γ increases the immune response by stimulating macrophages and natural killer cells. Interleukin (IL)-10 is an immune-regulatory cytokine that has both immunosuppressive and anti-angiogenic functions against tumors [37].

*Identification of suitable cytotoxic T-cell epitopes*

Based on their antigenicity, non-allergenicity, and nontoxic characteristics, three MHC class I-binding epitopes were identified in *LYN* out of the 938 epitopes. Similarly, three epitopes were identified in *AKT1* out of 2490 epitopes (Table 2A).

The 3D structures of the six MHC-1 epitopes were constructed using the PEP-FOLD server. Using ClusPro and HPEPDOCK web servers, every epitope was docked against TLR4. Servers assign scores based on their binding affinities. HSEKNVVYR, EVAHTLTENR, EVLEDNDYGR, DRARFYGAEIVSALD, ENLMLDKDGHIKITD, and KEKATGRYYAMKILKK have binding affinities of -166.444, -158.416, -144.018, -212.695, -174.264, and -222.914 kcal mol-1, respectively.

*Identification of suitable helper T-cell epitopes*

Of the 999 epitopes, two MHC class II-binding epitopes were identified in *LYN*, while only one epitope was identified in the *AKT1* protein out of 659 epitopes based on their high antigenic scores, low toxicity, and no allergenicity (Table 2B).

Three-dimensional structures of MHC class II epitopes were built using the PEP-FOLD server. To establish binding affinities, all epitopes were docked against TLR4 using default settings on ClusPro and HPEPDOCK servers. The epitopes DRARFYGAEIVSALD, ENLMLDKDGHIKITD, and EEMEVSLAKPKHRVT had binding affinities of -212.695, -174.264, and -198.663, respectively.

*Real time qPCR analysis of AKT1 and LYN*

To determine the significance of *AKT1* and *LYN* in liquid biopsy samples of oral cancers and healthy controls, we assessed the expression levels of these genes in serum samples. As shown in Figure 2a-2b, the expression of *AKT1* (control: 1.34 ± 0.43; Patients: 2.02 ± 0.77) and *LYN* (control: 1.23 ± 0.37; patients: 1.75 ± 0.79), was markedly higher in oral cancer samples than in normal controls.

HPAanalysis demonstrated that *AKT1* was localized to the nucleoplasm and microtubules, while *LYN* was localized to the plasma membrane, vesicles, and Golgi apparatus (Figure 3a). The analysis also revealed a

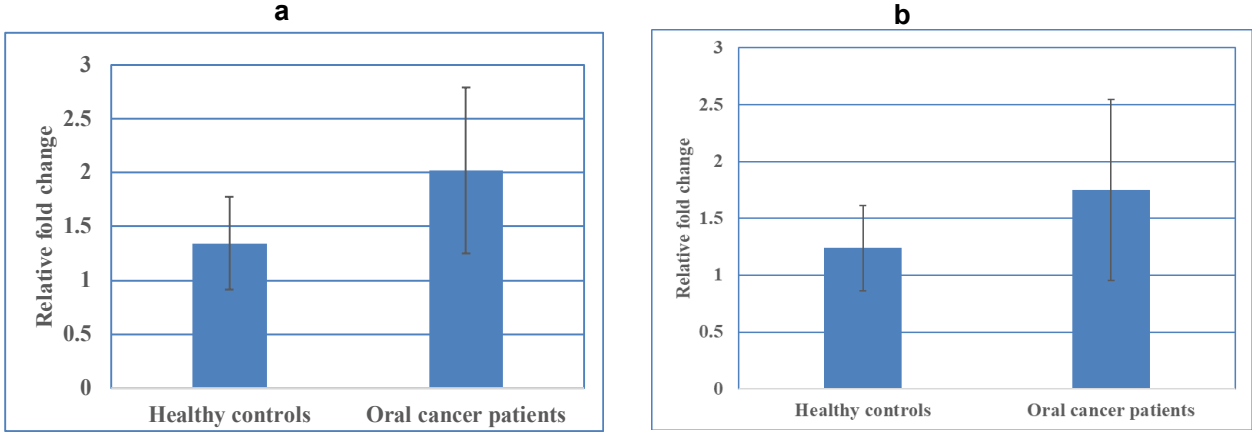


Figure 2. qPCR Analysis of a) *AKT1* and b) *LYN* Genes in Oral Cancer Samples and Normal Controls

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

| SN | MHC Class I binding epitopes | ALLELE (SMM) | ALLELE (NetMHCpan 4.1) | *AS | *PS | *PCS |
|---|---|---|---|---|---|---|
| 1 | HSEKNVVYR | HLA-C*05:01 | HLA-A*31:01 | | | |
| | | HLA-C*12:03 | HLA-A*33:01 | 0.77 | 1.6 | 1.02 |
| | | HLA-A*68:01 | HLA-A*11:01 | | | |
| | | HLA-A*31:01 | HLA-A*30:01 | | | |
| 2 | EVAHTLTENR | HLA-C*12:03 | HLA-A*33:01 | | | |
| | | HLA-C*03:03 | HLA-A*31:01 | 0.68 | 1.09 | 1.72 |
| | | | HLA-A*33:01 | | | |
| | | | HLA-A*31:01 | | | |
| 3 | EVLEDNDYGR | HLA-C*05:01 | HLA-A*33:01 | | | |
| | | HLA-C*12:03 | HLA-A*68:01 | 0.86 | 1.64 | 1.09 |
| | | | HLA-A*31:01 | | | |
| | | | HLA-A*33:01 | | | |
| 4 | KTWRPRYFLLK | HLA-A*32:01 | HLA-A*23:01 | | | |
| | | HLA-C*12:03 | HLA-B*58:01 | 1.13 | 1.16 | 0.85 |
| | | | HLA-A*30:01 | | | |
| | | | HLA-B*08:01 | | | |
| 5 | LLKKDPKQRLG | HLA-C*12:03 | HLA-A*31:01 | | | |
| | | HLA-C*12:03 | HLA-B*08:01 | 0.64 | 0.81 | 0.21 |
| | | HLA-C*12:03 | HLA-A*02:03 | | | |
| | | HLA-B*15:02 | HLA-B*08:01 | | | |
| 6 | NQDHEKLFEL | HLA-C*12:03 | HLA-B*08:01 | | | |
| | | HLA-C*05:01 | HLA-B*40:01 | 0.73 | 1.38 | 1.81 |
| | | | HLA-A*02:06 | | | |
| | | | HLA-B*08:01 | | | |

high proportion of patients expressing *AKT1* (patient proportion: 1) compared to *LYN* (patient proportion: 0.5) (Figure 3b).

*Antigenicity, allergenicity, solubility, and physicochemical evaluation of vaccine designs*

The vaccine was constructed by combining suitable B and T cell epitopes using linkers and adjuvant, as described in the Materials and Methods section. The threshold value for the antigen score was 0.5. The antigenic score of the vaccine was higher than the threshold value and was approximately similar either with (0.58) or without adjuvant (0.57). These results demonstrate the antigenicity of the vaccine construct. Furthermore, the vaccine was stable (instability index = 14.14), slightly polar in character (GRAVY value = -0.485), non-allergenic, and non-toxic. In this study, the vaccine construct showed a high aliphatic index, suggesting its thermal stability (Table 3).

The construct was soluble (>0.535), as predicted by Protein-Sol. These results suggest the suitability of this vaccine construct for oral cancer. The vaccine construct contained 57.5% helices (230 amino acids), 7.5% β-sheets (30 amino acids), and 35% coil turns (140 amino acids), as predicted by the PSIPRED server.

*Tertiary structure prediction, refinement and validation*

The I-TASSER server generates five three-dimensional models of the vaccine construct based on the best threading templates of the PDB library: 2acxA, 2bcjA, 3c4wB, 3c51B, 3nynA, 4myiA, 4tnbA, 4yhjA, 7t4tA, and 8em8A. The C-scores of these models ranged from -1.73 to -2.92.

Table 2B. List of Top Scoring MHC Class II Binding Helper T-cell Epitopes in *AKT & LYN*

| MHC Class II binding epitopes | Allele (SMM_ align method) | Allele (NetMHCIIpan-4.1) | AS* | Allergenicity | Toxicity |
|---|---|---|---|---|---|
| DRARFYGAEIVSALD | HLA-DRB1*01:01 | HLA-DQA1*01:02/DQB1*06:02 | 0.76 | | |
| | | HLA-DPA1*03:01/DPB1*04:02 | | NON-ALLERGEN | NON-TOXIC |
| ENLMLDKDGHIKITD | HLA-DRB3*01:01 | HLA-DPA1*03:01/DPB1*04:02 | 0.97 | | |
| | | HLA-DPA1*01:03/DPB1*04:01 | | NON-ALLERGEN | NON-TOXIC |
| EEMEVSLAKPKHRVT | HLA-DRB5*01:01 | HLA-DRB1*12:01 | 0.62 | | |
| | | HLA-DRB1*09:01 | | NON-ALLERGEN | NON-TOXIC |

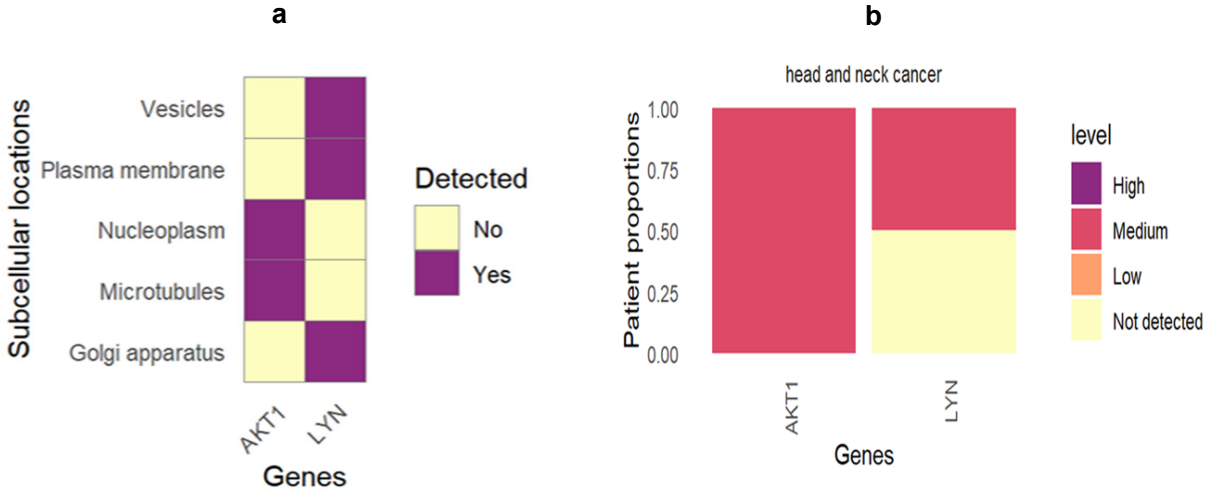


Figure 3. a) Subcellular localization of *AKT1* and *LYN* b) Patient proportions of *AKT1* and *LYN* in oral cancer. The data was analyzed using HPAanalyze R package in R studio.

A model with a high C-score was chosen for refinement. The original model was refined using GalaxyRefine server. Following refinement, five models were generated. The accuracy of these models was defined by GDA-HA, MolProbity, and poor rotamers. Model 3 was chosen based on the GDA-HA, MolProbity, and bad rotamer scores. In the improved model, GDT-HA was determined to be 0.9275, whereas MolProbity and poor rotamers were calculated to be 1.858 and 1, respectively. These scores were higher for the original structure (GDT-HA: 1, MolProbity score: 2.833, and poor rotamer: 18.1).

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 87.7%, 10.3%, 0.6%, and 1.4% of the residues were favored, permitted, generously allowed, and outlier areas, respectively, in the refined model. The ERRAT server was used to analyze the statistics of non-bonded interactions. The ERRAT score was set at 87.28 for the original model and 89.54 for the refined model. Generally, an ERRAT score greater than 50 represents a good quality model; thus, a score of 89.54 validates our modelled structure. ProSA-web showed the Z-score of the refined vaccine candidate as -5.4 in comparison to the original model (z score: -5.1).

*Molecular docking of vaccine construct with TLR4*

The vaccine design showed polar interactions with chains B and D of TLR4 (Table 4).

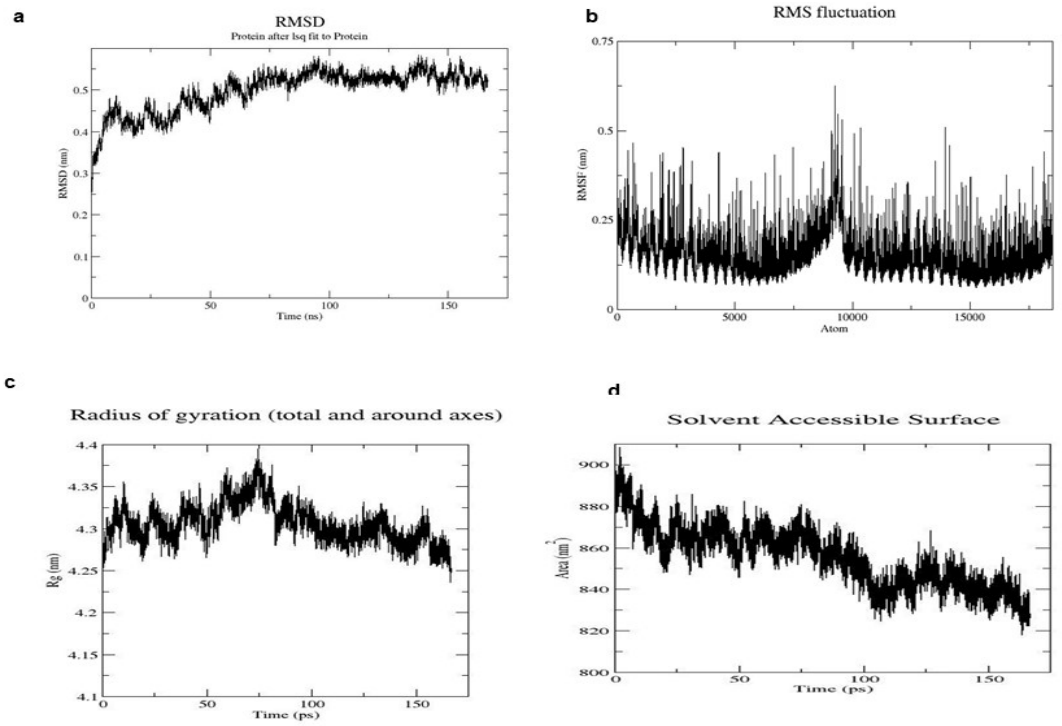


Figure 4. Molecular Dynamics Simulation Study of TLR4-Vaccine Construct. A) Root mean square deviation B) Root mean square fluctuations C) Radius of gyration D) Solvent accessible surface area for the time duration of 200 ns.

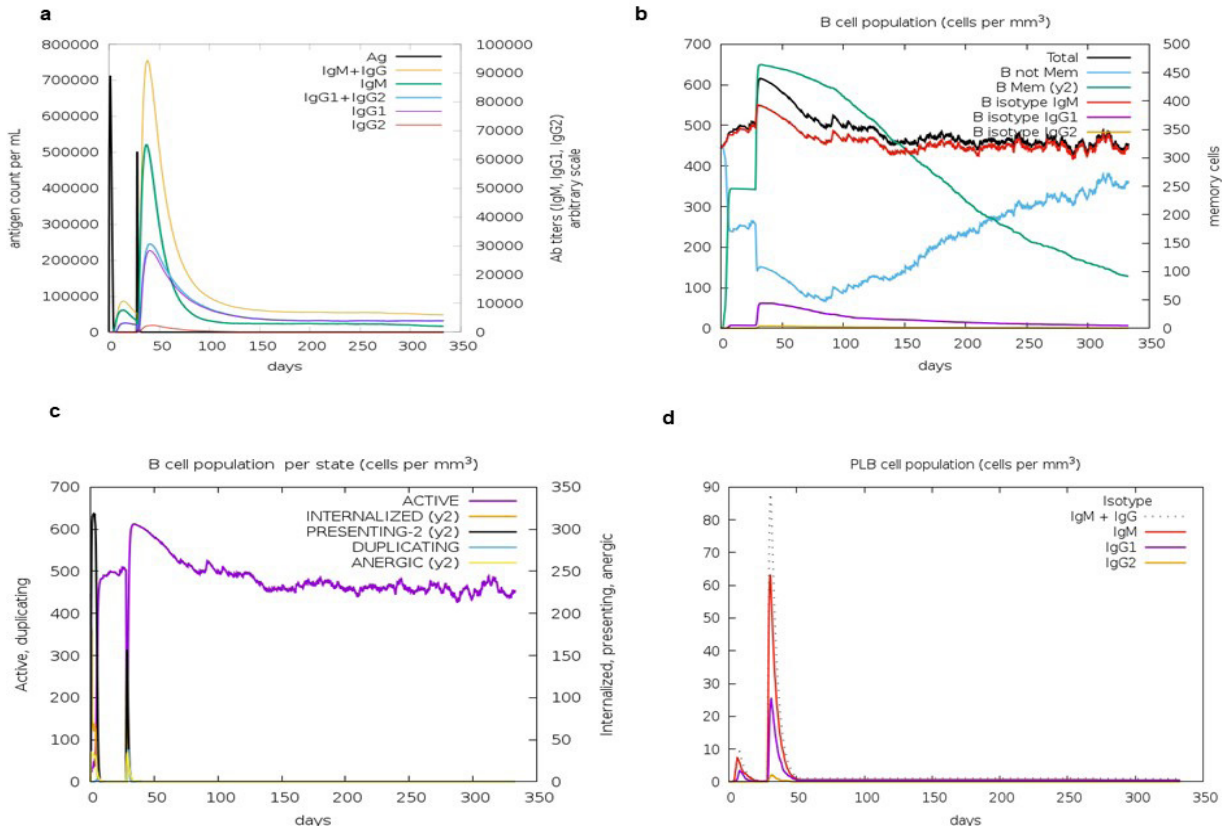


Figure 5. A) a Production of several subclasses of immunoglobulin (colored lines) in response to vaccine injection (black vertical lines. B) B cell population b PLB cell population C) B cell population per stated TH cell population D) PLB cell population.

*Molecular dynamics simulation*

The stability of the vaccine-TLR4 complex was evaluated using root mean square deviation (RMSD). The RMSD oscillates below 0.6 nm throughout the

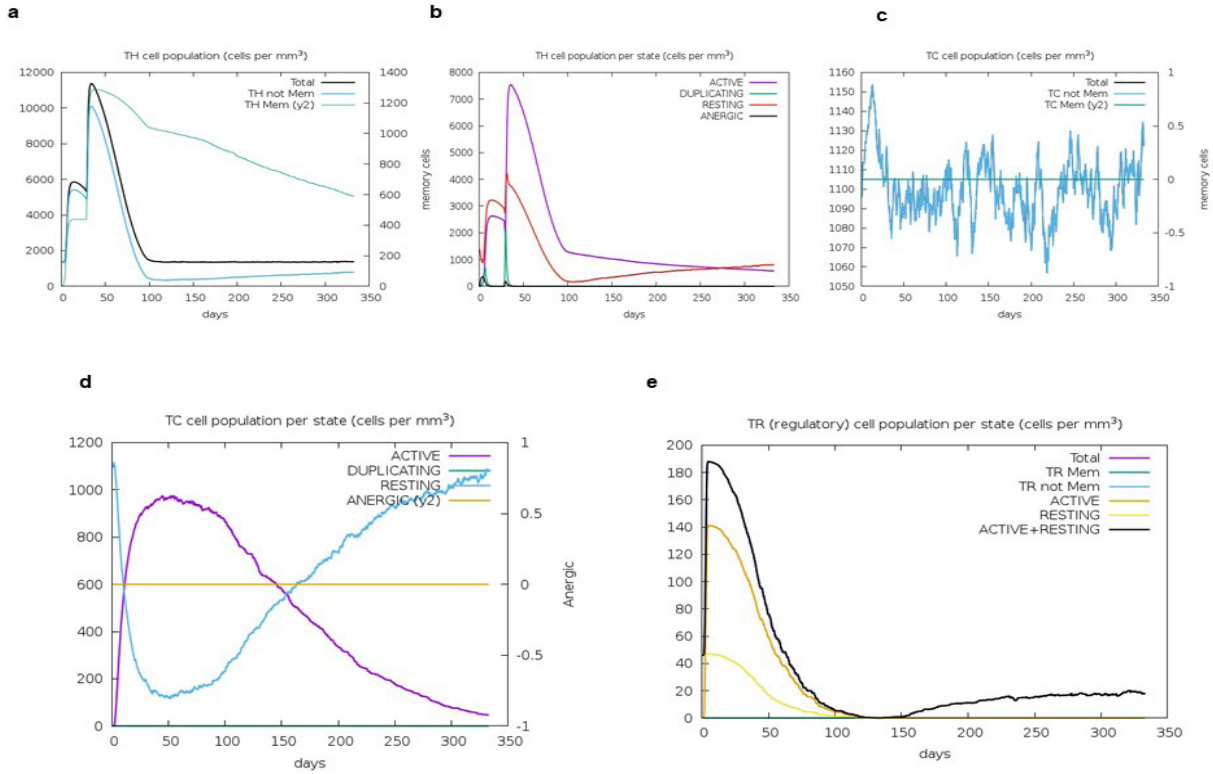


Figure 6. A) TH cell population B) TH cell population per state C) TC cell population D) TC cell population per state E) TR cell population

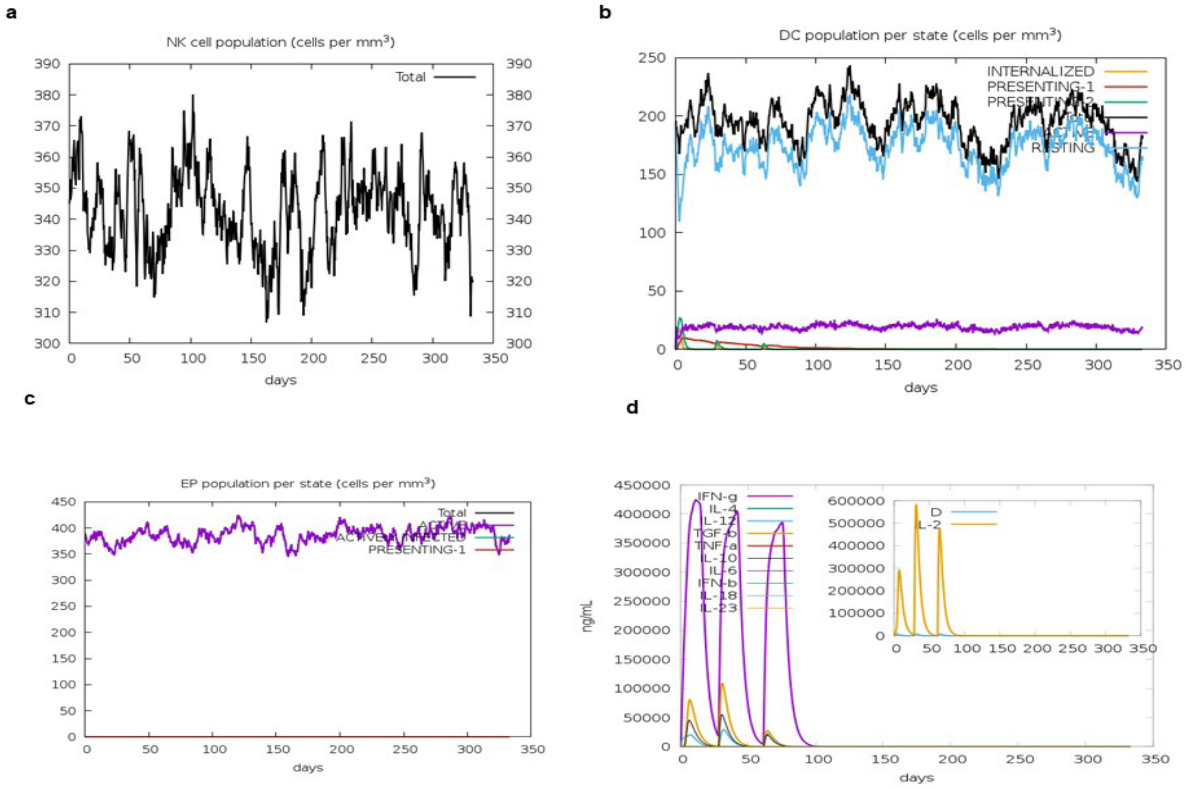


Figure 7. A) Natural killer cell population B) Dendritic cell population per state C) Epithelial cell population per state D) Production of Cytokine and Interleukin.

simulation of 150 ns (average: 0.49 ± 0.05) (Figure 4a). We previously found that chains B and D of TLR4 show polar interactions with the vaccine construct (Table 4). The RMSF plot revealed that there were less variations in the chain B (atom numbers: 5825 to 11689) and the chain D atoms (atom numbers: 13078-14465) of TLR4 (RMSF-chain B: 0.17 ± 0.07 SD; chain D: 0.13 ± 0.05 SD) (Figure 4b). The average predicted RMSF of the vaccine (atom numbers: 23745-28994) also had a small fluctuation (0.61 ± 0.0059) throughout the simulation. These results suggest fewer fluctuations in the residues of the receptor and vaccine constructs in the docked complex. The average value of radius of gyration was 4.27 ± 0.04 nm and it remained constant throughout the simulation,

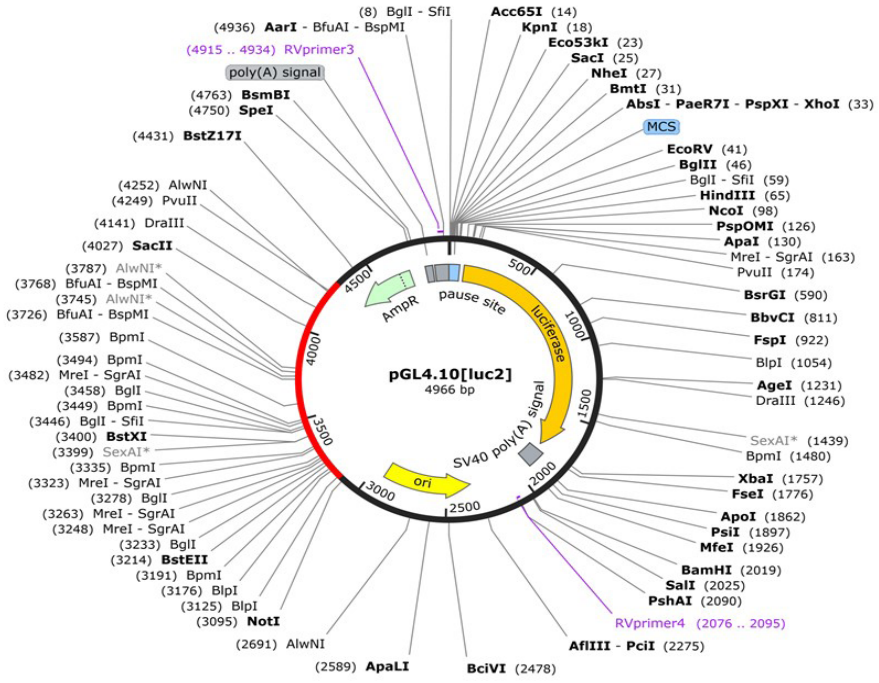


Figure 8. A Diagrammatic Map of *in silico* Gene Cloning.

Table 3. Evaluation of the Vaccine Construct

| Property | Value | | |
|---|---|---|---|
| Number of amino acids | 400 | | |
| Molecular weight | 44344.57 | | |
| Theoretical pI | 6.83 | | |
| Atomic composition | Carbon | C | 1994 |
| | Hydrogen | H | 3158 |
| | Nitrogen | N | 540 |
| | Oxygen | O | 593 |
| | Sulphur | S | 5 |
| Total number of negatively charged residues (Asp + Glu) | 64 | | |
| Total number of positively charged residues (Arg + Lys) | 63 | | |
| Formula | C1994H3158N540O593S5 | | |
| Extinction coefficients | 40800 | | |
| Estimated half-life | 30 Hours | | |
| Net-charge | -0.03 | | |
| Instability index | 14.14 | | |
| Aliphatic index | 82.9 | | |
| Grand average of hydropathicity (GRAVY) value | -0.485 | | |
| Allergenicity | Non-allergen | | |
| Toxicity | Non-Toxic | | |
| Solubility | 0.535 | | |

Table 4. Residues of Vaccine Forming Polar Interaction with *TLR4*

| SN | TLR4 residues | Vaccine residues |
|---|---|---|
| 1 | Chain B: N541 | E376 |
| 2 | Chain B: E31 | K79 |
| 3 | Chain B: N544 | E376 |
| 4 | Chain B: R227 | A38, P39 |
| 5 | Chain B: R598 | D71 |
| 6 | Chain B: E603 | G71 |
| 7 | Chain B: Q547 | E68 |
| 8 | Chain B: K477 | E143 |
| 9 | Chain B: Q523 | E136, H139 |
| 10 | Chain B: K477 | E143 |
| 11 | Chain B: E425 | R145 |
| 12 | Chain B: Q423 | K188 |
| 13 | Chain B: N448 | G307 |
| 14 | Chain B: S472 | G309 |
| 15 | Chain B: S471 | E310 |
| 16 | Chain B: Q333, K354 | E164 |
| 17 | Chain B: R355 | D135, N154 |
| 18 | Chain B: E287 | R158 |
| 19 | Chain B: E286 | K165, H162 |
| 20 | Chain D: E143 | K91 |
| 21 | Chain D: E144 | R82, L90 |

suggesting the compactness of the complex (Figure 4c). Similarly, the average value of SASA comes out to be 839.9 ± 13.3 nm2, suggesting that the hydrophobic core of the complex was exposed to the surrounding aqueous medium (Figure 4d). The results also showed that the electrostatic (-2980.9±178.9) and van der waals energies (-736.6±133.3) of the docked complex were negative, which ultimately resulted in a negative binding energy (-311.4±29.7 kcal/mol). All these evidences indicate strong binding affinity of vaccine to TLR4 during the 150 ns simulation.

*Characterisation of immune responses of the construct*

Simulated immunological responses showed higher levels of IgM + IgG, IgG1, IgG2, and memory B cells with antigen clearance (Figure 5a-5d).

The designed vaccine induced an immune response by activating multiple immune cells, including TH, TC, regulatory T cells, memory B cells, natural killer cells, dendritic cells, and cytokines, for a period of one year. Epithelial cells, which are involved in nonspecific immunity, have also been implicated (Figure 6a-6e, and 7a-7d).

*Codon adaptation of the vaccine construct*

The optimized codon had a length of 1150 nucleotides with 68.75% GC content (optimal range 30% to 70%), and a codon adaptation index (CAI) of 0.96. These values indicate the stable expression of the constructed vaccine in the E. coli strain. Furthermore, a recombinant plasmid was created by inserting an altered gene sequence into the pGL4.10 [luc2] vector, using the restriction enzymes *SacII* and *BstXI* (Figure 8).

## Discussion

Epitope-based vaccinations induce a certain immune response while averting adverse responses to unfavorable epitopes. Multi-epitope-based vaccines have numerous advantages such as enhanced safety profiles, comparatively easy administration, strong immunogenicity, and the production of a broad range of antibodies. However, multi-epitope vaccines show high levels of antigenic and immunogenic effects, in contrast to conventional single-epitope vaccines [3]. Multipeptide vaccines have been developed for the treatment of several cancers. For example, the IDM-2101 vaccine consists of 10 peptides with Montanide ISA51 and was used in phase I/II trials for the treatment of small cell lung carcinoma [38]. The vaccine demonstrates no or minimal adverse effects, with a median overall survival of 17.3 months. Similarly, another vaccine consisting of 13 synthetic peptides derived from prostate tumor antigens was used to treat prostate cancer patients [39]. Another multi-epitope vaccine, IMA901, demonstrated longer overall survival in patients with renal cancer [40]. However, no well-known vaccine has been designed to prevent oral cancer. Developing a vaccine against oral cancer is necessary to combat this life-threatening disorder.

Peptide cancer vaccines are designed based on suitable B and T cell epitopes that can elicit both humoral and

cellular immune responses against tumor-associated antigens or tumor-specific antigens. However, several issues remain in the design of safe and effective vaccines. For example, selection of suitable antigens, adjuvants, and immunization regimens is a major challenge. Using in silico computational approaches, these issues can be resolved with a high therapeutic efficacy and minimal side effects. In this study, two proteins, *LYN* Proto-Oncogene from the Src Family Tyrosine Kinase and *AKT1* (AKT Serine/Threonine Kinase 1), were identified as suitable vaccine candidates out of 54 antigens that were selected via a literature search and clinical trial sites. The high Pi score of both these genes indicates their high chances of success as suitable vaccine candidates. Both *LYN* and *AKT1* play an important central role in cellular growth, development, and survival of cancer [6, 9]. For example, it was shown that *AKT1* plays a central role in apoptosis via regulating Bcl2 family member proteins, survivin, and cyclin D1. Moreover, silencing of *AKT1* reduced tobacco-induced aggressiveness by inhibiting the proliferation and migration of oral squamous cell carcinoma [41]. Similarly, Src family kinases such as Lyn regulate proliferation, differentiation, apoptosis, migration and metabolism of cancer cells [8]. In addition, *LYN* overexpression has been reported in oral cancer and is considered a potential therapeutic target in treating oral cancer [9, 42]. Lyn kinase also mediates migration and tumor growth in EGF receptor variant III (EGFRvIII) expressing Head and Neck Cancer. EGFRvIII is a truncated growth factor receptor which show more aggressive behavior [43].

Vaccination enhances the immune system by targeting antigens linked to oral cancer cells. Identification of T- and B-cell epitopes is another important part of vaccine design. HTLs, CTLs, and adjuvants are the three components of peptide vaccination that are vital for cellular immune responses [44].Therefore, suitable B and T cell epitopes were identified based on their physiochemical attributes, allergenicity, and toxicity, using several bioinformatics tools. These MHC-I- and MHC-II-binding epitopes were predicted to be 9-mer and 15-mer peptides from *AKT1* and *LYN* proteins, respectively. The epitopes found in our study have a high affinity for major histocompatibility complex (MHC) molecules and robust antigenicity, which highlights their ability to elicit potent humoral and cellular immune responses. Next, a multi-epitope vaccine candidate was designed using linkers to join epitopes by integrating reverse vaccinology and computational approaches for maximal efficacy against oral cancer. Interestingly, while AAY linkers enhance epitope presentation, GPGPG patterns boost HTL immunity. The EAAAK protein domain distinguishes these proteins [5].

50S ribosomal protein L7/L12 was chosen as the adjuvant because of its capacity to interact with TLR4. There are many justifications for the activation of TLR4 in relation to cancer vaccination. First, TLR4 interacts with APCs, such as neutrophils and natural killer (NK) cells, and promotes the recruitment and activation of innate immune effector cells into the tumor microenvironment [45]. These two types of cells are dendritic cells (DCs) and macrophages. These cells enhance the immune response to malignancies by destroying tumor cells and increasing the secretion of proinflammatory cytokines. Co-stimulatory molecules, such as CD80 and CD86, are elevated on APCs as a result of TLR4 signaling. Effective T cell activation and differentiation depends on these substances. Thus, tumor-associated antigens (TAAs) are strongly opposed by CTLs. Notably, the 50S ribosomal proteins L7/L12 and human TLR4 are agonistic. Therefore, it was included in the N-terminal site of the vaccine [46]. The aliphatic index of the vaccine was 82.90, suggesting that the vaccine was hydrophobic, with increased thermostability. This vaccine is stable, non-allergic, and non-toxic in nature.

The 3D structure of the vaccine construct was refined using GalaxyRefine server. The server produced a refined model via side-chain optimization and energy minimization to minimize steric conflicts and improve the local geometry. Subsequently, the refined model underwent an even more rigorous refinement process involving energy reduction and extensive molecular dynamics simulations to further improve the overall structural quality. To evaluate the refined models, GalaxyRefine uses several metrics, such as the Root Mean Square Deviation (RMSD) values compared to the original model and the MolProbity score, which measures the stereochemical quality of protein structures, as well as the clash score. The accuracy of the model was determined using both the MolProbity and clash scores. A decline in MolProbity and rotamer scores was noted when the revised model was compared with the original model [47]. The Ramachandran plot revealed that 98% of the amino acids were either in the most favored portions or permitted regions. These results demonstrate that the quality of the vaccine construct was satisfactory.

The vaccine candidate had a robust affinity for the TLR4 receptor, as demonstrated by the polar interactions between them. Strong interaction with TLR4 is essential for eliciting an amazing immune response against oral cancer. TLR4 agonists have been used in various cancer vaccines, including oral cancer [48-51]. The results of the molecular dynamics simulation indicated low fluctuations in RMSD and Rg, suggesting its stability and compactness during the 150 ns simulation. The negative value of the binding energy also suggested a high binding affinity of the vaccine to TLR4. Immune-simulation studies have shown high levels of IgG/IgM, IgG1, and IgG2 with activation of various immune cells and antigen clearance. Although these findings are promising, the study has limitations. In future, the vaccine should be evaluated in cellular and animal models. If the results are promising in terms of producing humoral and cell mediated immunity in xenograft models, it can be proceeded to conduct clinical trials. It is important to emphasize that further animal and cellular culture studies are needed to confirm the effectiveness of this vaccine in greater detail before initiating clinical trials.

In conclusion, in this study, 54 genes were screened using various bioinformatics tools to develop a multiepitope peptide-based vaccine for oral cancer. These 54 genes were identified using extensive literature searches on various websites such as PubMed, MEDLINE, Google Scholar, and ClinicalTrials. Gov (last accessed dates: 13-11-2023). Next, the vaccine was constructed by linking various B

and T-cell epitopes and adjuvants via linkers. The epitopes were selected based on their physicochemical attributes, antigenicity, allergenicity, toxicity, and immunogenicity. An agonist of TLR4 was used as an adjuvant. Toll-like receptors (TLRs) induce anti-cancer immunity. Several clinical trials and multiple reports have reported the efficacy of TLR agonists as adjuvants to chemotherapeutic drugs, radiation, immunotherapies, and cancer vaccines, including TLR4 agonists [52, 53, 49, 48]. It has been reported to TLR4 be involved in the eradication of oral cancer by OK-432 [50, 51]. OK-432 is a lyophilized form of penicillin-killed Streptococcus pyogenes that has been used as an immunotherapeutic agent against oral cancer [50]. The results of molecular simulations suggest a strong binding affinity between the vaccine and the TLR4 receptor. These results suggest suitability of the vaccine for oral cancer

## Author Contribution Statement

Samir Shaikh, Daksh Kunchala, Drishti Velecha, Suresh Prajapati: Methodology and data analysis. Manasi Patel: Collection of samples, qPCR.Reeshu Gupta: Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

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*Declaration of competing interest*

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.

*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.

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