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چنداپی توپی لنفوسیت T سیتوتوکسیک با هدف گیری پروتئین های
غشایی در سرطان معده

***In Silico* Design and Structural Validation of a Multi-Epitope Cytotoxic T Lymphocyte Vaccine Candidate Targeting Membrane-Associated Proteins in Gastric Cancer**

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Abstract

Gastric cancer (GC) is still among the top causes of death from cancer globally. Immunotherapy, in the form of cytotoxic T lymphocyte (CTL) vaccines against tumor-specific antigens, is a promising therapeutic approach. The effective design of multi-epitope vaccines with high immunogenicity, tumor specificity, and structural stability, however, is not straightforward. The study aimed to develop and structurally validate a multi-epitope CTL vaccine targeting membrane-associated proteins overexpressed in GC, utilizing integrated immunoinformatics and structural vaccinology approaches. Experimentally verified 9-mer CTL epitopes were selected from five GC membrane proteins. Immunogenicity was enhanced and antigen processing facilitated by the addition of β -defensin 3 adjuvant and suitable linkers. Vaccine construct physicochemical property analysis, prediction of allergenicity and toxicity, three-dimensional structure modeling, Ramachandran plot validation, and molecular docking into HLA-A*0201 molecules were done. The vaccine developed possessed the desirable physicochemical characteristics of high stability, non-allergenicity, and non-toxicity. Confirmation of structure revealed 87.8% residues in favorable spaces, ascertaining correct folding and structural integrity. Molecular docking analysis revealed strong binding affinity (-874.2 kcal/mol) with HLA-A*0201, indicating efficient MHC-I presentation and the capacity to generate a good CTL response. The *in silico* rationalized multi-epitope CTL vaccine possesses promising immunogenic and structural characteristics as a personalized GC immunotherapeutic lead. Further studies, including molecular dynamics simulations, broader HLA allele coverage, and experimental validation, are warranted to confirm efficacy and safety and facilitate clinical translation.

Keywords: Gastric cancer, CTL vaccine, Epitope, Immunoinformatics, Docking

چکیده

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خلاصه

سرطان معده همچنان یکی از علل اصلی مرگومیر ناشی از سرطان در جهان است. ایمنی درمانی، به‌ویژه در قالب واکسن‌های لنفوسیت T سیتوتوکسیک (CTL) علیه آنتی‌ژن‌های اختصاصی تومور، رویکرد درمانی امیدبخشی محسوب می‌شود. با این حال، طراحی مؤثر واکسن‌های چنداپی‌توپی با ایمن‌زایی بالا، اختصاصیت توموری و پایداری ساختاری مناسب، فرایندی پیچیده است. این مطالعه با هدف توسعه و اعتبارسنجی ساختاری یک کاندید واکسن CTL چنداپی‌توپی علیه پروتئین‌های غشایی بیش‌بیان‌شده در GC، با استفاده از رویکردهای یکپارچه ایمونوانفورماتیک و واکسن‌شناسی ساختاری انجام شد. اپی‌توپ‌های ۹ اسیدآمینه‌ای CTL که به‌طور تجربی تأیید شده بودند، از پنج پروتئین غشایی مرتبط با GC انتخاب شدند. برای افزایش ایمن‌زایی و تسهیل پردازش آنتی‌ژن، ادجوانت بتا‌دفنسین ۳ و رابط‌های مناسب به سازه واکسن افزوده شد. تحلیل ویژگی‌های فیزیکوشیمیایی، پیش‌بینی آلرژی‌زایی و سمیت، مدل‌سازی ساختار سه‌بعدی، اعتبارسنجی با نمودار رامچاندرا و داکینگ مولکولی با HLA-A*0201 انجام گرفت. واکسن طراحی‌شده دارای پایداری بالا و فاقد آلرژی‌زایی و سمیت بود. نتایج ساختاری نشان داد ۸/۸۷٪ از آمینواسیدها در نواحی مطلوب قرار دارند. داکینگ مولکولی تمایل اتصال قوی (۸۷۴/۲- کیلوکالری بر مول) با HLA-A*0201 را نشان داد که بیانگر ارائه کارآمد توسط MHC-I و توان القای پاسخ CTL مناسب است. واکسن CTL چنداپی‌توپی طراحی‌شده، ویژگی‌های ایمن‌زایی و ساختاری مطلوبی به‌عنوان یک گزینه ایمنی درمانی شخصی‌سازی‌شده برای GC دارد. مطالعات تکمیلی برای تأیید کارایی و ایمنی ضروری است.

کلیدواژه‌ها: سرطان معده، واکسن CTL، اپی‌توپ، ایمونوانفورماتیک، داکینگ

مقدمه

Introduction

Gastric cancer (GC) represents a significant global health burden, ranking among the leading causes of cancer death across the world. Despite advancements in diagnostic and therapeutic strategies, the outlook for GC remains poor, largely due to diagnosis at advanced stages, extensive molecular and histological heterogeneity, and limited efficacy of conventional treatments. Current studies emphasize the central role of the tumor microenvironment, cancer stem cells, and epigenetic alterations in GC development, which culminate in immune evasion, metastasis, and chemotherapy resistance. These complexities necessitate the integration of newer biotechnology approaches, such as multi-omics profiling, liquid biopsy-based biomarkers, and personalized immunotherapeutic regimens, to enhance early detection, refine prognostic assessments, and enhance treatment response (Yang et al., 2023).

Recent advancements in immunotherapy have shifted cancer treatment from non-specific cytotoxic therapy to immune-based specific treatment. Peptide vaccines, especially those targeting cytotoxic T lymphocyte (CTL) responses, have shown promise in killing tumor cells selectively. In GC, membrane-bound neopeptides are highly specific and immunogenic. Membrane-associated proteins are attractive targets for *in silico* vaccine candidate design due to their tumor-specific expression and surface accessibility, facilitating effective CTL recognition. Computational tools now enable precise prediction and modeling of histocompatibility complex class I (MHC-I)-binding peptides. This is in favor of the rational design of multi-epitope vaccines tailored to the tumor's immunologic profile (Ashi et al., 2022). The success of CTL-based vaccines entirely depends on the discovery of immunodominant epitopes with high accuracy-short antigenic peptides with stable binding to MHC-I molecules and effective recognition by T cell receptors (TCRs), triggering vigorous antigen-specific immune responses (Baruah & Bose, 2020).

Recent developments in immunoinformatics and structural vaccinology have also facilitated the *in silico* identification of immunodominant T-cell epitopes using the integration of sequence homology, MHC-binding affinity prediction, and population coverage analysis. These high-throughput computational systems leverage machine learning algorithms and structural modeling to strengthen antigen selection and epitope mapping with improved accuracy. This approach, therefore, significantly expedites vaccine design pipelines by lessening the reliance on time-consuming and resource-intensive

experimental screening (Prawiningrum et al., 2022). Tumor-associated antigens, including membrane proteins overexpressed in cancer cells, can be processed and presented by MHC class I molecules to be recognized by cytotoxic T lymphocytes. Selective expression in tumors provides the possibility of vaccine therapy with minimal off-target effects (Miao et al., 2023). In GC, several well-characterized membrane proteins, including HER2 (ERBB2), MET, CDH1 (E-cadherin), EGFR, and CEACAM5, have been implicated in tumor development, invasion, and metastasis (Lordick et al., 2014; Zhang et al., 2022). While their therapeutic targeting with monoclonal antibodies and kinase inhibitors has been explored, their exploitation as sources of immunodominant CTL epitopes for multi-epitope vaccine development remains to be fully harnessed.

Despite growing focus on peptide-based cancer vaccines, there is a vast knowledge gap between the combination of epitope prediction, structural modeling, and functional validation in GC. Most studies have focused on one epitope or employed limited prediction criteria, which can lead to suboptimal immunogenicity and translational irrelevance.

The objective of this study is to rationally design a multi-epitope cytotoxic T lymphocyte (CTL) GC Candidate vaccine through an end-to-end immunoinformatics and structural vaccinology pipeline (Oghbatalab et al., 2020; Abbasi & Masoudi-Nejad, 2020). GC is a leading cancer-causing death, with little success in conventional treatment and the need for urgently immunologically targeted therapy. To meet this challenge, we focused on a panel of experimentally validated, membrane-bound proteins that are overexpressed by GC cells. Overlapping 9-mer peptides were generated from these proteins and fully screened against the HLA-A*02:01 allele based on predicted MHC class I binding affinity, immunogenicity, physicochemical properties, and structural stability. Strong epitopes were selected and grouped into a chimeric vaccine structure, consisting of an immunostimulatory adjuvant β -defensin 3 and cleavable AAY linkers to enable increased antigen processing. The 3D structure of the vaccine was modeled and optimized, and docking simulations against the HLA-A*0201 molecules were conducted to assess binding interactions.

This research is to offer a computationally verified, structurally stable vaccine candidate, paving the way towards future in vitro and in vivo verification and potentially participating in personalized immunotherapy regimens for GC treatment.

Materials and Methods

مواد و روش‌ها

Methodological procedure, comprising 10 major steps, is described in detail in later sections and graphically presented in Fig 1.

1. Protein Selection and Data Collection

All computations were conducted under the R programming environment (version 4.4.2) aided by a series of packages like rentrez (v1.2.3), Biostings (v2.74.1), dplyr (v1.1.4), and stringr (v1.5.1) (Skoulakis et al., 2025; Wickham, 2020; Winter, 2017). A comprehensive dataset of proteins participating in GC was initially manually collected from the literature and validated further with UniProt Knowledgebase annotations. Only membrane-bound proteins that were found to be of concern to GC were selected. Subcellular localization and functional annotation were retrieved from UniProt to ensure cell surface expression, maximizing relevance for CTL-based vaccine design (Bateman et al., 2025; Mohammadi, Safaralizadeh, & Asadi, 2025).

2. Epitope Generation and Immunogenicity Profiling

Full-length protein sequences were dissected into overlapping 9-mer peptides with a sliding window strategy to ensure accurate representation of HLA-A*02:01-restricted epitopes. The peptides were subsequently subjected to extensive physicochemical analysis using Peptides (v2.4.6), seqinr (4.2-36), and tidyverse (v2.0.0) R packages (Osorio et al., 2015; Wickham, 2021). These parameters were hydrophobicity, net charge, isoelectric point (pI), instability index, aliphatic index, and Boman index. A propriety scoring algorithm was utilized to make predictions of the immunogenicity of each peptide. Peptides with instability index < 40 and immunogenicity scores within the top 25th percentile were chosen as high-confidence CTL candidates (Mohammadi, Safaralizadeh, & Safarzadeh, 2025; Mohammadi & Safaralizadeh, 2025; Mohammadi & Safaralizadeh, 2025).

3. Selection of Final Epitopes for Vaccine Design

Last epitope selection was performed on multi-parameter screening on the basis of the estimated MHC binding affinity and immunogenicity potential. The peptides with IC50 values less than 500 nM in simulated conditions were high-affinity candidates (Zhao & Sher, 2018). Among these, epitopes with more immunogenicity scores (≥ 2.1) were prioritized, alongside examination of critical physicochemical features like negative net charge and moderate hydrophobicity (Islam et al., 2025). Five peptides from four GC-associated proteins were finally selected, the best combination of binding ability, immunogenicity, and structural stability. These prospects were reserved for continued structural modeling and vaccine construct development.

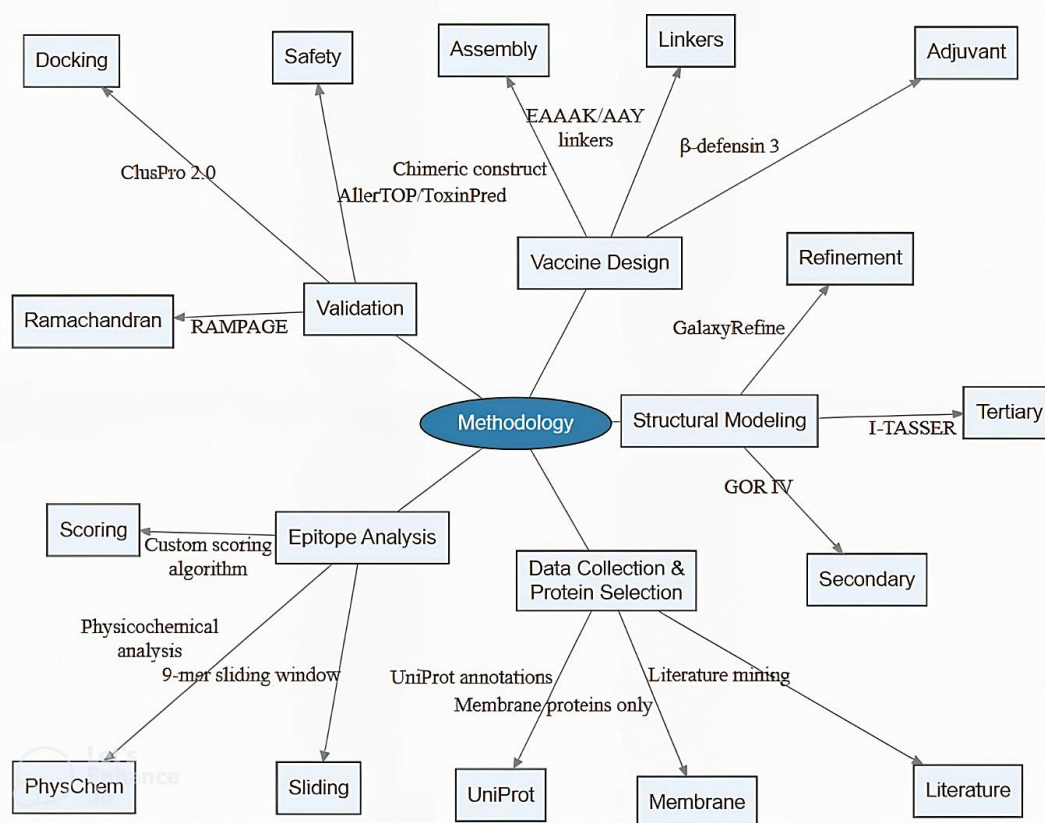


Fig 1. Workflow of computational vaccine design, showing key steps from data sampling and epitope examination to structural modeling (I-TASSER, GalaxyRefine) and verification (ClusPro 2.0, RAMPAGE). Adjuvant (β -defensin 3) and linker (EAAAK/AAY) incorporation are depicted.

4. Selection of Final Epitopes

The ultimate candidate CTL epitopes were identified based on combining immunogenicity scores, physicochemical properties, and experimentally confirmed MHC binding affinity through simulated data. The combined criteria included high immunogenicity (top 25th percentile), low instability index (< 40), negative GRAVY values, and high MHC binding (Khazaei et al., 2024).

5. Multi-Epitope Vaccine Construct Design

The selected CTL epitopes were assembled into a single linear polypeptide. An N-terminal β -defensin 3 adjuvant was added to enhance immunogenicity. A fixed EAAAK linker was used to maintain structural independence between the adjuvant and epitope region. All of the CTL epitopes were linked with AAY spacers for efficient proteasomal processing and MHC class I presentation. The terminal sequence was saved in FASTA format for future structural modeling (Nezafat et al., 2016; Pandey et al., 2018).

6. Secondary Structure and Disorder Prediction

The predicted construct's secondary structure was predicted using the GOR IV method available through the NPS@ server. α -helix, β -sheet, and coil predictions were performed based on statistics-based models and alignment-derived parameters (Gamier et al., 1996). Intrinsically disordered regions were predicted by IUPred2A with a disorder threshold score of 0.5 (Mészáros et al., 2018).

7. Prediction of Tertiary Structure and Refinement

Tertiary structure prediction was performed using the I-TASSER web server. The program uses threading and iterative refinement to construct probable 3D structures. Among the models generated, the best model with the highest confidence scores was used for structural refinement (Yang et al., 2015). GalaxyRefine was utilized to improve structural quality through side-chain repacking and energy minimization (Heo et al., 2013). Refinement model was checked based on typical measures like MolProbity score, clash score, and Ramachandran plot statistics.

8. Structural Validation

Ramachandran plot analysis was carried out with the RAMPAGE server to check for stereochemical quality. Residues were categorized under favored, allowed, and outlier regions based on their ϕ and ψ angles. The model was considered valid for subsequent analysis if it satisfied pre-defined structural criteria (Laskowski et al., 1993).

9. Physicochemical Analysis

The final vaccine construct was examined for biochemical properties using the ExPASy ProtParam tool and PepCalc. Parameters screened were molecular weight, isoelectric point (pI), extinction coefficient, instability index, GRAVY score, and net charge at physiological pH. These calculations helped determine the construct's suitability for expression, stability, and solubility in physiological conditions (Gasteiger et al., 2005). Allergenicity and toxicity of the whole multi-epitope vaccine construct, including the linker and adjuvant regions, were predicted using the AllerTOP v2.1 and ToxinPred servers, respectively. These analyses were carried out on the full amino acid sequence of the ultimate vaccine construction in FASTA format (Dimitrov et al., 2014; Gupta et al., 2013).

10. Molecular Docking with MHC Class I

To model interactions of the vaccine with MHC class I receptor, docking was performed with ClusPro 2.0. The Balanced energy model was used to include van der Waals, electrostatics, and desolvation effects. Multiple binding poses were constructed and clustered, and the most populated and energetically favorable one was chosen for visualization and interface analysis using PyMOL (Schrodinger, 2015; Vajda et al., 2017).

Results and Discussion

نتایج و بحث

1. Candidate Protein Identification and Epitope Generation

The 1,090 overlapping 9-mer peptides were generated from ten GC -associated membrane proteins (Table 1). Based on physicochemical evaluation and a custom immunogenicity scoring model, peptides with an instability index below 40 and immunogenicity scores in the top 25th percentile were identified as high-confidence CTL epitope candidates.

Table 1. List of gastric cancer-associated proteins investigated in this study. All proteins are primarily located at the cell membrane.

GeneName	ProteinID	ProteinName
ERBB2 (Lordick et al., 2014)	P04626	Receptor tyrosine-protein kinase erbB-2
CDH1 (Lordick et al., 2014)	P12830	Cadherin-1
MUC1 (Kim et al., 2023)	P15941	Mucin-1
CEACAM5 (Zhang et al., 2022)	P06731	Carcinoembryonic antigen-related cell adhesion molecule 5
HER2 (Lordick et al., 2014)	P04626	Receptor tyrosine-protein kinase erbB-2
CLDN18	P56856	Claudin-18
MET (Lordick et al., 2014)	P08581	Hepatocyte growth factor receptor
FGFR2 (Lordick et al., 2014)	P21802	Fibroblast growth factor receptor 2
KRAS (Lordick et al., 2014)	P01116	GTPase KRas
EGFR (Lordick et al., 2014)	P00533	Epidermal growth factor receptor

2. Binding Affinity and Epitope Validation

Of the 1,090 putatively predicted 9-mer peptides, 84 had high binding affinity to MHC class I, with $IC_{50} < 500$ nM. Of these, five of the epitopes—DNADDEVDT and NADDEVDT (MET), DHVRENRR (HER2), DDEDDTDGA (FGFR2), and ELDREDFEH (CDH1)—were chosen based on reproducible performance across simulated binding affinity tests. The DNADDEVDT epitope had the most significant predicted interaction with an IC_{50} of 177.93 nM and is a good candidate as a promising CTL. This pool enhanced self-confidence in the immunogenic and presentation potential of chosen peptides (Fig 2, Table 2).

Table 2. Prioritized CTL epitopes for gastric cancer vaccine design, showing predicted IC50, immunogenicity, charge, and hydrophobicity (GRAVY).

No.	Gene	Pro.ID	Epitope	IC50 (nM)	Imm. Score	Charge	GRAVY
1	MET	P08581	DNADDEVDT	177.93	2.3	-5	-1.74
2	HER2	P04626	DHVRENRRGR	292.66	2.14	+1.09	-2.6
3	FGFR2	P21802	DDEDDTDGA	321.83	2.53	-6	-2.26
4	CDH1	P12830	ELDREDFEH	484.85	2.16	-3.9	-2.07
5	MET	P08581	NADDEVDT	489.18	2.14	-3	-1.86



Fig 2. A. Hydrophobicity frequency plot (GRAVY scores) of GC-related membrane protein candidate epitopes. More negative GRAVY scores indicate higher hydrophilicity, which is preferable for immunogenicity. The graph illustrates epitopes from proteins such as Cadherin-1, Claudin-18, and Receptor tyrosine-protein kinase erbB-2, illustrating their physicochemical heterogeneity. **B.** MHC-I predicted binding affinity distribution (IC50 values in nM) in filtered peptides. Peptides were separated into high-affinity binders (IC50 < 500 nM, dark bars) and low-affinity binders (IC50 ≥ 500 nM, light bars).

3. Construction of Vaccine Constructs and Structural Modeling

A five-epitope vaccine construct was designed by joining the five selected CTL epitopes with AAY linkers to boost proteasomal processing and MHC class I presentation. An N-terminal β -defensin 3 adjuvant was joined via a rigid EAAAK linker to trigger innate immunity and establish structural distance from the epitope domain. The final construct had 107 amino acids. Secondary structure prediction by GOR IV algorithm provided a structure with 38.3% α -helices, 15.9% β -strands, and

45.8% random coils. IUPred2A analysis further indicated that the sequence did not include large stretches of intrinsically disordered regions, indicating structural order and potential for folding (Fig. 3).

The final vaccine construct combines an immunostimulatory adjuvant with multiple CTL epitopes, supporting its potential as a gastric cancer vaccine candidate. The full amino acid sequence of the designed construct is shown below, with linker regions indicated:

GIINTLQKYYCRVRGGRCVLSCLPKEEQIGKCSTRGRKCCRRKK-EAAAK-DNADDEVDT-AAAY-DHVRENRRGR-AAAY-DDEDDTDGA-AAAY-ELDREDFEH-AAAY-NADDEVDT

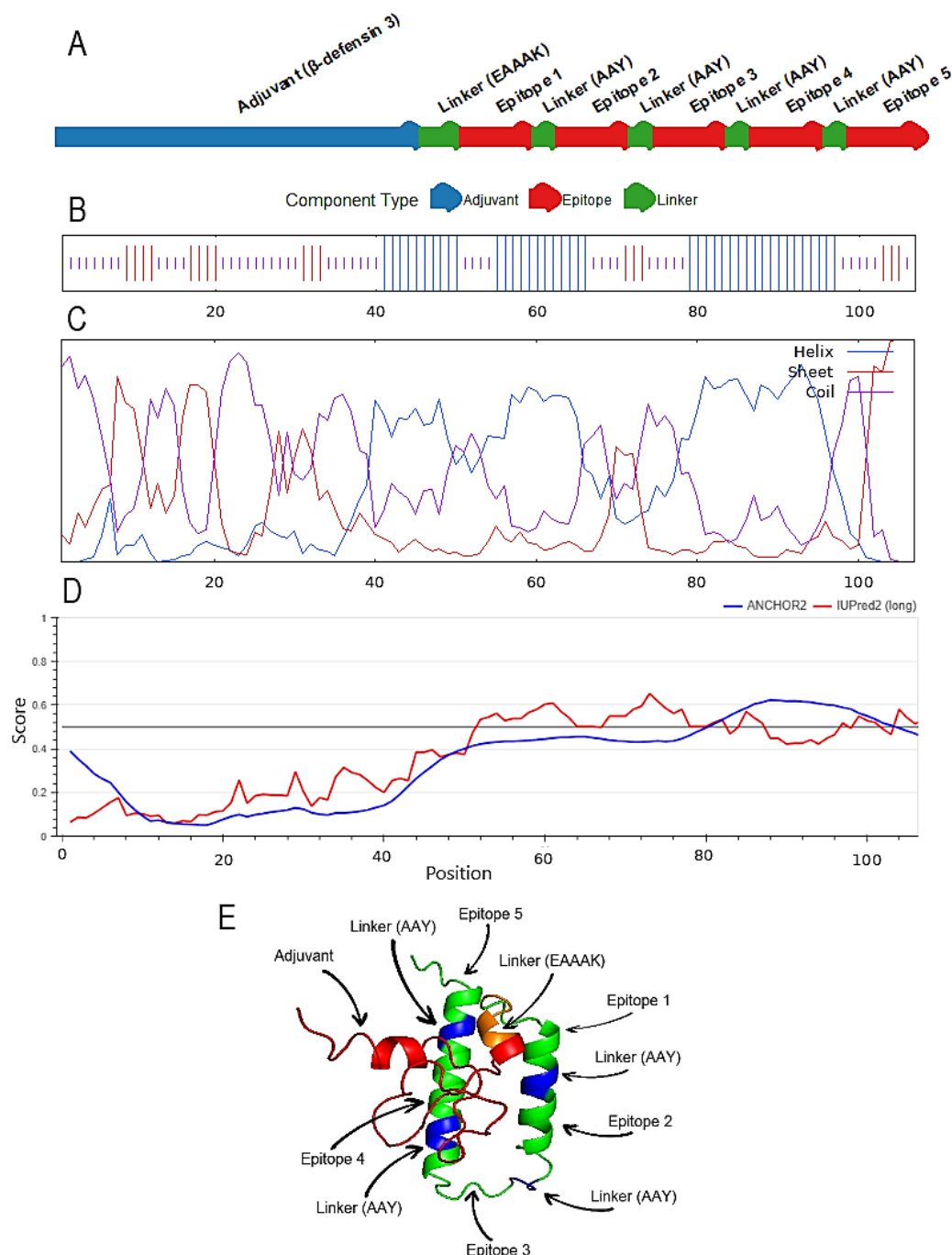


Fig 3. A: Schematic illustration of the multi-epitope vaccine construct, illustrating the N-terminal β -defensin 3 adjuvant (blue), EAAAK linker (green), and CTL epitopes (red) joined by AAY linkers (green). The design is optimized for proteasomal processing and MHC-I presentation. **B & C:** Secondary structure predictions show a composition of 38.3% α -helices (blue), 15.9% β -sheets (red), and 45.8% random coils (magenta) indicates a balanced structural profile. **D:** Disorder propensity analysis indicates negligible disordered regions (scores <0.5), supporting the stability of the construct. **E:** 3D model demonstrates compact folding with epitopes oriented for optimal immune recognition.

4. Validation and Biochemical Characterization

Structural verification of the improved model was done using RAMPAGE. Ramachandran plot analysis indicated 87.8% residues in most favored regions, 10.2% in additionally allowed regions, and 2.0% in generously allowed regions, with no residues in disallowed conformations. Although somewhat lower than the 90% benchmark for high-resolution models, the absence of outliers indicated acceptable stereochemical quality for downstream applications.

Physicochemical characteristics of the construct were also analyzed using ProtParam and PepCalc. The predicted molecular weight was 12.07 kDa, and the theoretical pI ranged from 4.63 to 4.94. The instability index was 23.15, and thus the construct fell under the stable category. The GRAVY score of -1.112 and net charge of -6.2 at pH 7 suggested strong hydrophilicity and good solubility in water. These data confirmed that the designed construct meets the key biochemical requirements of antigenicity, solubility, and stability (Fig. 4A, 4B).

Allergenicity prediction using AllerTOP v2.1 predicted the vaccine construct as a probable non-allergen. ToxinPred analysis demonstrated that all CTL epitopes chosen were non-toxic and that no toxic motif existed within the core immunogenic region. Potentially toxic stretches were limited to arginine-rich segments within the β -defensin 3 adjuvant region.

5. Protein-MHC Docking Analysis

To evaluate the interaction of the vaccine construct modeled with the human MHC class I molecule, molecular docking simulations were performed using ClusPro 2.0. The reference docking structure was the HLA-A*0201/CD8/viral peptide complex (PDB ID: 1AKJ), a well-characterized, high-resolution (2.65 Å) Homo sapiens MHC class I glycoprotein.

From the generated docking models, Cluster 0 was selected based on its large population size ($n = 138$) and highly favorable energy scores. The most negative binding energy observed was -874.2 kcal/mol, while that of the center model was -727.6 kcal/mol, indicating a highly stable and energetically favorable interaction.

PyMOL visualization affirmed that the vaccine construct docked comfortably into the peptide-binding groove of the HLA-A2 molecule. The construct formed tight, stable contacts with no steric clashes, and the spatial arrangement of epitopes suggested optimal surface exposure for CTL recognition and antigen presentation. Use of the 1AKJ structure enhanced the validity of docking predictions and allowed close inspection of epitope-MHC interactions in a biologically relevant setting (Fig. 4C, 4D).

Discussion: In this study, we designed a multi-epitope cytotoxic T lymphocyte (CTL) vaccine candidate for gastric cancer using an integrated immunoinformatics and structural vaccinology approach. The construct consists of five high-confidence 9-mer epitopes derived from experimentally validated, membrane-associated proteins overexpressed in gastric cancer, combined with an N-terminal β -defensin 3 adjuvant and rationally selected linker sequences. These design elements were incorporated to enhance immunogenicity, optimize proteasomal processing, and improve antigen presentation. The rigid, helix-forming EAAAK linker was employed to maintain structural separation between functional domains, whereas the AAY linker was used to facilitate efficient epitope cleavage and MHC class I presentation.

Conformational stability of the vaccine was established through structural validation by Ramachandran plot analysis and molecular docking simulations, along with high binding affinity to the HLA-A*0201 molecules, showing its promise as an personalized immunotherapy candidate for GC.

The selected CTL epitopes were very immunogenic, having suitable binding affinities for effective MHC-I presentation. They also have good physicochemical properties—low instability indexes and negative GRAVY scores—promoting stability and immunogenicity. The β -defensin 3 adjuvant was included to promote innate immune activation through TLR signaling without the introduction of allergenicity or toxicity, as verified by AllerTOP and ToxinPred prediction. Structural inspection revealed 87.8% of residues within favored Ramachandran regions, and docking simulations indicated good binding energy with HLA-A*0201, which is suggestive of excellent receptor affinity.

Several recent studies have used immunoinformatics tools to design cancer vaccines; our work has unique strengths in tumor specificity and structural rigor, though. Unlike Shojaeian et al. (2023), who focused on epitope prediction against *Helicobacter pylori*, our research sought to membrane-associated GC-specific tumor antigens with better clinical relevance. We adopted a robust R-based workflow that involved peptide scoring, physicochemical filtering, and epitope selection followed by extensive 3D structure modeling, Ramachandran plot examination, and MHC-I docking. Despite Shojaeian et al. adding preliminary experimental steps, their *in silico* design did not include structural intensity captured in our pipeline (Shojaeian et al., 2023).

Similarly, Yazdani et al. (2022) developed a colorectal cancer vaccine with good antigenicity and epitope prediction but did not incorporate detailed structural modeling or MHC-binding validation. Our method, on the other hand, enhances immunological precision by incorporating physicochemical criteria, structural optimization, and MHC-I docking simulations (Yazdani & Rafiei, 2022).

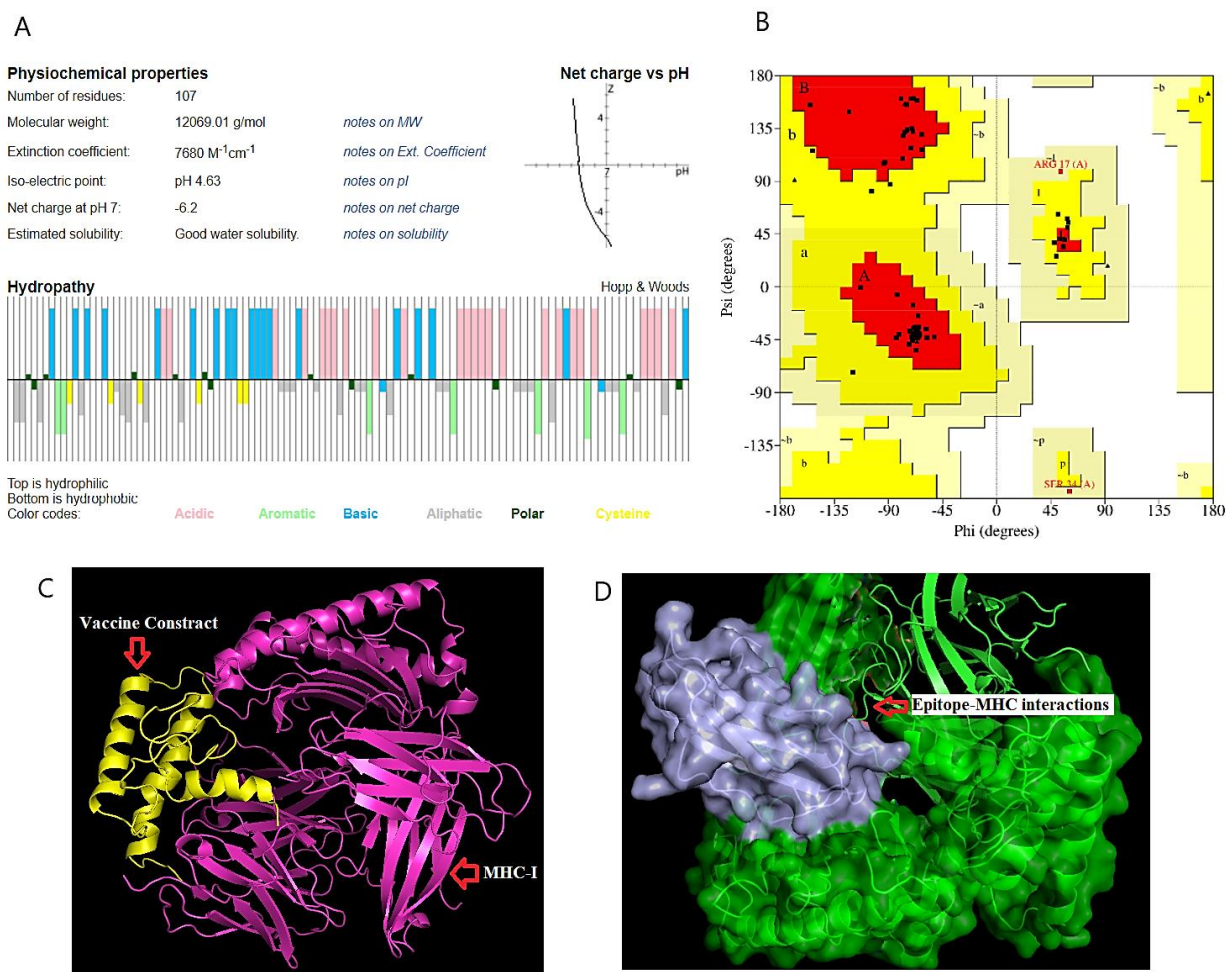


Fig 4. A: Key physicochemical properties of the vaccine construct **B:** Ramachandran plot validation indicates 87.8% favorable residues (dark blue), without outliers, indicating robust structure stability. **C:** Interaction of the vaccine construct with MHC-I highlighting stable binding within the peptide groove. **D:** Epitope-MHC interactions (red) with significant hydrogen bonds and favorable binding energy (-874.2 kcal/mol).

Sanami et al. (2021) designed a therapeutic HPV vaccine against oncoproteins E6/E7, including allergenicity analysis, molecular docking, MD simulations, and *in silico* cloning. While post-docking dynamic validation was included in their pipeline, missing in ours at this point—their antigen selection did not involve the tumor-specificity that is typical of our membrane protein-based approach (Sanami et al., 2021).

Alam et al. (2025) also constructed a multi-epitope vaccine for PD-L1, utilizing CTL, HTL, and B-cell epitopes, with MD simulations and immune response modeling. While their addition of more advanced immune components and simulation-predicted immunogenicity contributes to the added functional knowledge, our vaccine offers better structural screening and tumor antigen specificity. However, the absence of immune simulation and helper/B-cell epitope incorporation in the present work may limit its immunological reach (Mahafujul Alam et al., 2025).

Finally, Dariushnejad et al. (2022) constructed a recombinant TNBC vaccine against CEA, MTDH, and MUC-1 epitopes, along with a TLR4 agonist, and validation by docking and MD simulations. Increased epitope strategy and receptor-level validation are predictive of immune activation potential. Our work, however, offers a more stringent epitope selection pipeline and thorough MHC-I interaction analysis, even though without dynamic and multi-epitope class engagement (Dariushnejad et al., 2022). While previous multi-epitope cancer vaccine designs have made important contributions, some have relied on less stringent epitope selection or have not fully emphasized tumor-specific membrane localization and antigen processing considerations. In our study, we sought to build upon these foundations by applying a careful filtering strategy, focusing on membrane-associated proteins, and performing detailed MHC-I binding analysis, aiming to enhance the potential translational relevance of the proposed vaccine candidate.

Limitation: Our study is still in its infancy amid these promising outcomes. The absence of molecular dynamics simulations restricts insight into long-term stability and mobility of the vaccine-MHC complex in physiological environments. Furthermore, sole focus on CTL epitopes without HTL or B-cell components may potentially limit the breadth and longevity of

immunity. Experimental validation *in vitro* and *in vivo* to confirm immunogenicity, cytotoxicity, and safety is also necessary. Restricting MHC binding predictions to HLA-A*0201 alone may reduce vaccine utility in genetically diverse populations.

Subsequent research will have to encompass HTL and B-cell epitopes to elicit a more extensive immune response by stimulating humoral and cellular immunity. Expanding epitope prediction beyond a single HLA allele will increase population coverage and translation applicability. Conducting molecular dynamics simulations will provide better understanding of conformational dynamics and vaccine stability. Finally, empirical evaluation with cellular assays and animal models will be required for clinical translation.

Conclusion: We describe here a rationally engineered multi-epitope cytotoxic T lymphocyte (CTL) vaccine for GC, designed through an integrative immunoinformatics and structural vaccinology strategy. By selecting five high-affinity epitopes from overexpressed membrane-bound proteins in GC and incorporating a β -defensin 3 adjuvant, the vaccine construct is promising in its immunogenicity. Careful structural validation using Ramachandran plot analysis and molecular docking with HLA-A*0201 ensured that the vaccine was stable and had good receptor binding affinity. The physicochemical profiling validated the best stability, non-allergenicity, and non-toxicity, boosting its compatibility for immunotherapeutic applications.

While this *in silico* study provides a robust basis for epitope selection and structural optimization, limitations such as absence of molecular dynamics simulations, low HLA allele coverage, and absence of experimental validation are future areas to be addressed. Extension of epitope prediction into multiple HLA alleles and incorporation of helper T-cell and B-cell epitopes can enhance the efficacy and scope of the immune response. Furthermore, empirical confirmation using *in vitro* and *in vivo* assays is important to ensure the immunogenicity and safety profile of the vaccine.

In conclusion, the proposed vaccine is a strong candidate for personalized GC immunotherapy with modular design scalability to other types of cancers. Its discovery proves the strength of computational vaccinology in accelerating precision cancer vaccine development, the path to improved therapeutic interventions.

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Authors Contribution: Kianoush Mohammadi performed the analyses and wrote the manuscript. Seyedeh Zahra Bakhti designed the study, supervised the work, and revised the manuscript. Both authors read and approved the final manuscript.

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