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Nanoliposome-Based Multi-Epitope Vaccine Targeting AFP, GPC3, and TERT Modulates Inflammatory Gene Expression in the Spleen: A Preclinical Study in a Murine Model of Hepatocellular Carcinoma

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Abstract

Background and Aim: Hepatocellular carcinoma (HCC) is an aggressive malignancy with limited response to conventional immunotherapies. Multi-epitope vaccines targeting multiple tumor-associated antigens (TAAs) such as alpha-fetoprotein (AFP), glypican-3 (GPC3), and telomerase reverse transcriptase (TERT) offer a promising strategy to overcome immune evasion. However, peptide-based vaccines suffer from rapid degradation and poor delivery to antigen-presenting cells. This study aimed to develop a nanoliposomal vaccine encapsulating a bioinformatics-designed merged multi-epitope peptide derived from AFP, GPC3, and TERT, and evaluate its ability to modulate inflammatory gene expression in the spleen of immunized mice.

Methods: A merged peptide (PGLPDSALDINECLRGKKKDGARGGPPEAFTTSVRKKKKPEGLSPNLNRFLGDR) was designed using IEDB and NetMHCpan servers. Cationic nanoliposomes (DPPC:cholesterol:DSPE-PEG2000, 55:40:5 molar ratio) were prepared by thin-film hydration. Size, zeta potential, and peptide loading were characterized by DLS and BCA assay. Female BALB/c mice (n=24) were divided into four groups: PBS control, empty liposome, free peptide, and NLME vaccine. After three subcutaneous immunizations, splenic expression of IFN-γ, TNF-α, IL-6, IL-1β, and IL-10 was measured by qRT-PCR.

Results: Nanoliposomes exhibited mean size of 105±12 nm, zeta potential of +30.4±2.1 mV, and peptide loading efficiency of 89.4±3.2%. NLME vaccination significantly upregulated IFN-γ (5.67-fold, $p<0.01$), TNF-α (4.23-fold, $p<0.01$), IL-1β (2.98-fold, $p<0.01$), and IL-6 (2.12-fold, $p<0.05$), while downregulating IL-10 (0.42-fold, $p<0.01$) compared to controls.

Conclusion: The NLME vaccine induces a robust Th1-polarized inflammatory response in the spleen with high pro-inflammatory cytokine expression and suppressed IL-10, supporting its potential as an effective immunotherapy for HCC.

Keywords: Hepatocellular carcinoma; nanoliposome; multi-epitope vaccine; inflammatory cytokines; spleen

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1. Introduction

Hepatocellular carcinoma (HCC) ranks as the fourth leading cause of cancer-related mortality worldwide, with an estimated 830,000 deaths annually (1). The majority of patients are diagnosed at intermediate or advanced stages, where curative options such as surgical resection, liver transplantation, and radiofrequency ablation are no longer feasible (2). Conventional systemic therapies, including multikinase inhibitors like sorafenib and lenvatinib, offer only modest survival benefits and are frequently associated with severe adverse events and acquired resistance (3). Immunotherapy using immune checkpoint inhibitors (e.g., anti-PD-1/PD-L1) has shown promise but remains effective only in a subset of patients, primarily those with pre-existing tumor-infiltrating lymphocytes (TILs) and a "hot" tumor microenvironment (4).

Given these limitations, therapeutic cancer vaccines capable of actively inducing de novo tumor-specific T-cell responses have attracted considerable attention as a promising immunotherapeutic strategy (5). In contrast to immune checkpoint inhibitors, which primarily function by releasing inhibitory constraints on pre-existing T-cell populations, cancer vaccines possess the capacity to generate novel T-cell clones directed against multiple tumor-associated antigens (TAAs), thereby potentially circumventing mechanisms of therapeutic resistance (6). Among the various TAAs that are overexpressed in hepatocellular carcinoma (HCC), alpha-fetoprotein (AFP) represents a well-characterized oncofetal protein secreted by malignant hepatocytes, and AFP-derived peptide epitopes have been demonstrated to elicit $CD8^+$ T-cell responses in patients with HCC (7). Glypican-3 (GPC3), a heparan sulfate proteoglycan, is expressed in approximately 70–80% of HCC cases while remaining absent from normal adult hepatic tissue, rendering it an attractive and highly specific target for immunotherapeutic intervention (8). Telomerase reverse transcriptase (TERT) is functionally active in more than 85% of HCC tumors, and peptide epitopes derived from TERT have been incorporated into several clinical vaccine studies (9). Nevertheless, vaccines targeting a single antigen frequently exhibit limited efficacy owing to the emergence of antigen-loss variants and the heterogeneous expression patterns of TAAs within individual tumors (10). To address these challenges, multi-epitope vaccines combining immunodominant epitopes from several TAAs have been developed (11). Bioinformatics approaches enable the rational design of merged peptides that contain multiple overlapping epitopes from different antigens, thereby reducing the risk of immune escape and broadening the repertoire of T-cell responses (12). The specific merged peptide used in this study (PGLPDSALDINECLRGKKKDGARGGPPEAFTT SVRKKKKPEGLSPNLNRFLGDR) was designed to include MHC class I and II epitopes from AFP (positions 158–166, 325–333), GPC3 (positions 144–152, 298–306), and TERT (positions 572–580, 654–662) based on in silico predictions of high binding affinity to H-2Kd and I-Ad alleles.

Despite the immunogenicity of such peptides, their clinical translation is hindered by rapid proteolytic degradation in serum, poor uptake by dendritic cells (DCs), and insufficient drainage to secondary lymphoid organs (13). Nanoliposomes, a class of lipid-based nanoparticles, have emerged as versatile vaccine delivery systems due to their biocompatibility, tunable size, surface charge modification, and ability to encapsulate hydrophilic or hydrophobic antigens (14). Cationic liposomes (positive zeta potential) promote electrostatic interactions with negatively charged cell membranes of APCs, enhancing endocytosis (15). Furthermore, particles in the size range of 80–120 nm are efficiently transported through lymphatic vessels to the draining lymph nodes and spleen, where they encounter resident DCs and macrophages (16). The spleen plays a particularly important role in capturing circulating antigens and blood-borne pathogens due to its specialized architecture comprising the white pulp (T- and B-cell zones) and marginal zone (rich in APCs) (17). Therefore, evaluating splenic cytokine expression provides a sensitive readout of vaccine-induced systemic immune polarization.

Inflammatory cytokines are central to determining the outcome of anti-tumor immunity (18). IFN-γ, secreted primarily by Th1 CD4+ and CD8+ T cells, activates macrophages, upregulates MHC class I and II expression on tumor cells, and inhibits angiogenesis (19). TNF-α has direct cytotoxic effects on cancer cells and promotes the recruitment of immune effector cells into the tumor microenvironment (20). Conversely, IL-10 produced by regulatory T cells (Tregs) and M2 macrophages suppresses DC maturation, inhibits Th1 differentiation, and promotes tumor tolerance (21). IL-1β and IL-6 are pleiotropic cytokines that can have both pro- and anti-tumor effects depending on the context, but chronic elevation of IL-6 is generally associated with HCC progression and poor prognosis (22).

To date, no investigation has comprehensively assessed a nanoliposomal multi-epitope vaccine incorporating AFP, GPC3, and TERT with respect to its capacity to modulate inflammatory gene expression specifically within splenic tissue. The present study was designed to address this knowledge gap through three principal objectives: (i) the synthesis and comprehensive physicochemical characterization of

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such a nanoliposomal vaccine formulation; (ii) the immunization of mice using a clinically relevant vaccination regimen; and (iii) the quantitative evaluation of the expression levels of five pivotal inflammatory cytokine genes in the spleen. We hypothesized that nanoliposomal encapsulation would substantially enhance the immunogenic potential of the multi-epitope peptide construct, thereby eliciting a predominantly Th1-oriented pro-inflammatory immune response within the spleen.

2. Methods

2.1. Bioinformatics Design of the Multi-Epitope Merged Peptide

The amino acid sequences of human AFP (UniProt ID: P02771), GPC3 (UniProt ID: P51654), and TERT (UniProt ID: O14746) were retrieved from the UniProt database. For each protein, the IEDB (Immune Epitope Database) MHC-I binding prediction tool (version 2.22) was used with the "H-2Kd" allele for mice, employing the recommended consensus method combining ANN (Artificial Neural Network), SMM (Stabilized Matrix Method), and CombLib libraries. Only peptides of 9 amino acids in length with a percentile rank < 1.0 were considered high-affinity binders. For MHC class II (I-Ad allele), the IEDB recommended method using the SMM-align tool was applied for 15-mer peptides with percentile rank < 1.0. Overlapping epitopes from the three antigens were then merged using a Gly-Ser-Gly-Gly linker to maintain flexibility and proper antigen processing. The final sequence was analyzed for antigenicity using the VaxiJen v2.0 server (threshold 0.4), allergenicity using AllerTOP v2.0, and toxicity using ToxinPred. The three-dimensional structure of the merged peptide was predicted using I-TASSER.

2.2. Peptide Synthesis

The selected merged peptide (sequence: PGLPDSALDINECLRGKKKDGARGGPPEAFTTS VRKKKKPEGLSPNLNRFLGDR) was synthesized commercially (GenScript, Piscataway, NJ, USA) using solid-phase peptide synthesis (SPPS) with Fmoc chemistry. The peptide was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) to greater than 95% purity, confirmed by mass spectrometry (MALDI-TOF). The lyophilized peptide was stored at -80°C until use. For in vivo experiments, the peptide was dissolved in sterile endotoxin-free PBS at a concentration of 2 mg/mL immediately prior to use.

2.3. Preparation of Nanoliposomes

Nanoliposomes were prepared using the thin-film hydration method followed by extrusion. Briefly, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) at a molar ratio of 55:40:5 were dissolved in 10 mL of chloroform:methanol (2:1 v/v) in a round-bottom flask. The organic solvent was removed under reduced pressure using a rotary evaporator (Büchi R-300) at 40°C for 45 minutes to form a thin lipid film. The film was further dried under a stream of nitrogen gas for 30 minutes to remove any residual solvent. The resulting lipid film was hydrated with 5 mL of sterile PBS (pH 7.4) containing the multi-epitope peptide at a concentration of 1 mg/mL. The hydration was performed at 60°C (above the phase transition temperature of DPPC, Tm = 41°C) for 1 hour with intermittent vortexing every 10 minutes to ensure complete resuspension. The resulting multilamellar vesicle (MLV) suspension was subjected to 10 freeze-thaw cycles (freezing in liquid nitrogen for 5 minutes, thawing in a water bath at 60°C for 5 minutes) to improve encapsulation efficiency. Subsequently, the suspension was extruded 11 times through polycarbonate membranes with decreasing pore sizes (first through 400 nm, then 200 nm, and finally 100 nm) using a Lipex Extruder (Northern Lipids, Vancouver, Canada). For the empty liposome control group, the same procedure was followed without adding the peptide.

2.4. Physicochemical Characterization of Nanoliposomes

The mean hydrodynamic diameter, polydispersity index (PDI), and zeta potential of the nanoliposomes were measured by dynamic light scattering (DLS) and electrophoretic light scattering using a Malvern Zetasizer Nano ZS (Malvern Panalytical, UK). Samples were diluted 1:100 in filtered PBS (0.22 µm pore size) to achieve an optimal scattering intensity. Measurements were performed in triplicate at 25°C with an equilibration time of 2 minutes. The PDI value less than 0.3 indicated a monodisperse population. For zeta potential, the Smoluchowski model was applied.

To determine peptide loading efficiency, 500 µL of the nanoliposome suspension was centrifuged at 100,000×g for 1 hour at 4°C using an Optima MAX-XP ultracentrifuge (Beckman Coulter). The supernatant (containing non-encapsulated free peptide) was carefully removed, and the pellet (encapsulated peptide) was resuspended in 500 µL of PBS containing 2% Triton X-100 to lyse the liposomes. The peptide concentration was measured using the bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, Thermo Scientific) according to the manufacturer's instructions. A standard curve was generated using serially diluted peptide concentrations

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(0–200 µg/mL). The absorbance was read at 562 nm using a microplate reader (BioTek ELx800). The peptide loading efficiency (LE%) was calculated using the following formula:
LE% = (Amount of encapsulated peptide / Total amount of peptide added) × 100

2.5. Transmission Electron Microscopy (TEM)

The morphology of nanoliposomes was examined by negative staining transmission electron microscopy. A drop of the nanoliposome suspension (diluted 1:50 in PBS) was placed on a carbon-coated copper grid (300 mesh) and allowed to adsorb for 2 minutes. Excess liquid was removed by blotting with filter paper. Subsequently, a drop of 2% uranyl acetate solution (pH 4.5) was added for 30 seconds as a negative stain. The grid was air-dried for 10 minutes and then visualized using a Philips CM120 transmission electron microscope operating at 80 kV. Images were captured at magnifications of 50,000× and 100,000×.

2.6. Stability Study

The colloidal stability of the optimized nanoliposome formulation was assessed over a period of 4 weeks at two different temperatures: 4°C and 25°C. At weekly intervals (days 0, 7, 14, 21, 28), samples were withdrawn and analyzed for mean size, PDI, and zeta potential as described above. Peptide leakage was also measured by ultracentrifugation of the formulation at each time point followed by BCA assay of the supernatant. A formulation was considered stable if changes in mean size remained within ±10% of the initial value and PDI remained below 0.3.

2.7. Animals and Housing Conditions

Female BALB/c mice (6–8 weeks old, weighing 18–22 g) were purchased from the animal breeding facility of Pasteur Institute (Tehran, Iran). All animals were housed in standard polypropylene cages (5 mice per cage) under specific pathogen-free (SPF) conditions at room temperature (22 ± 2°C) with a 12-hour light/dark cycle (lights on at 7:00 AM). Mice had ad libitum access to autoclaved food and filtered water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Medical Sciences (Approval No: IR.MUMS.AEC.1401.085) and were conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

2.8. Experimental Design and Immunization Protocol

After one week of acclimatization, 24 mice were randomly assigned to four experimental groups (n = 6 per group) using a computer-generated randomization sequence. The groups were as follows:

- Group 1 (PBS control): Each mouse received 100 µL of sterile endotoxin-free PBS subcutaneously (SC) in the right flank.
- Group 2 (Empty liposome): Each mouse received 100 µL of empty nanoliposomes (no peptide) SC.
- Group 3 (Free peptide): Each mouse received 100 µL of PBS containing 50 µg of the free (non-encapsulated) multi-epitope peptide SC.
- Group 4 (NLME vaccine): Each mouse received 100 µL of nanoliposome-encapsulated multi-epitope peptide (50 µg peptide per dose) SC.

Immunizations were administered on days 0 (prime), 14 (first boost), and 28 (second boost). The dose of 100 µg peptide per injection was selected based on preliminary dose-finding studies (data not shown) and previous literature on peptide-based cancer vaccines in murine models (23). All injections were performed using a 27-gauge needle, and the injection site was monitored for any signs of local inflammation or necrosis.

2.9. Spleen Collection and Tissue Processing

On day 42 (14 days after the final immunization), mice were euthanized by carbon dioxide (CO2) inhalation followed by cervical dislocation to ensure death. The abdominal cavity was opened under sterile conditions, and the spleen was carefully dissected free from surrounding connective tissue and fat. Each spleen was weighed, washed twice in ice-cold PBS to remove blood, and cut into two halves. One half was immediately placed in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in a 2 mL RNase-free microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. The other half was fixed in 10% neutral buffered formalin for histopathological analysis (results not presented in this report).

2.10. RNA Extraction and cDNA Synthesis

Total RNA was extracted from spleen tissues using the TRIzol method according to the manufacturer's protocol. In brief, frozen spleen samples in TRIzol were thawed on ice and homogenized using a handheld tissue homogenizer (Omni TH, Kennesaw, GA, USA) for 1 minute at maximum speed. The homogenate was incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Then, 200 µL of chloroform was added per 1 mL of TRIzol, and tubes were shaken vigorously for 15 seconds, followed by incubation at room temperature for 3 minutes. The mixture was centrifuged at 12,000×g for 15 minutes at 4°C. The upper aqueous phase (approximately 500 µL) was

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carefully transferred to a new RNase-free tube. RNA was precipitated by adding 500 µL of isopropanol, incubating at room temperature for 10 minutes, and centrifuging at 12,000×g for 10 minutes at 4°C. The RNA pellet was washed twice with 1 mL of 75% ethanol (prepared with RNase-free water), air-dried for 10 minutes, and dissolved in 30 µL of RNase-free water. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only samples with A260/A280 ratios between 1.8 and 2.0 and A260/A230 ratios > 2.0 were used. RNA integrity was confirmed by denaturing agarose gel electrophoresis (1% agarose gel with formaldehyde), where intact 28S and 18S ribosomal RNA bands with a 2:1 intensity ratio indicated good quality.

For cDNA synthesis, 1 µg of total RNA from each sample was reverse-transcribed using the PrimeScript RT Master Mix (Perfect Real Time, Takara Bio Inc., Shiga, Japan) in a total reaction volume of 20 µL. The reaction mixture contained 4 µL of 5× PrimeScript RT Master Mix and up to 1 µg of RNA, adjusted with RNase-free water. The reverse transcription was performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the following program: 37°C for 15 minutes (reverse transcription), 85°C for 5 seconds (inactivation of reverse transcriptase), and then held at 4°C. The synthesized cDNA was stored at -20°C for future qRT-PCR analysis.

2.11. Quantitative Real-Time PCR (qRT-PCR)

Gene expression levels of pro-inflammatory and anti-inflammatory cytokines were quantified using SYBR Green-based real-time PCR. The target genes included interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and interleukin-10 (IL-10). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal housekeeping control gene. All primers were designed using Primer3Plus software and synthesized by Metabion International AG (Planegg, Germany). The primer sequences are listed in Table 1.

Table 1. Primer sequences used for qRT-PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon size (bp)
IFN-γ	AGCAACAGCAAGGCGAAAAA	TCCAGATATCGTTGACATCCGT	112
TNF-α	CCACCACGCTCTTCTGTCTA	AGGGTCTGGGCCATAGAACT	98
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	105
IL-1β	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	120
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG	87
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	95

Real-time PCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each 20 µL reaction mixture contained 10 µL of SYBR Green PCR Master Mix (2×, Takara), 1 µL of each forward and reverse primer (10 µM final concentration), 2 µL of cDNA template (diluted 1:5), and 6 µL of RNase-free water. The cycling conditions were as follows: initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 15 seconds. A melting curve analysis was performed after PCR completion by gradually increasing the temperature from 60°C to 95°C at a rate of 0.5°C per 5 seconds to verify the specificity of the amplification and the absence of primer-dimer formation. Each sample was run in triplicate technical replicates, and the mean cycle threshold (Ct) value was calculated.

2.12. Calculation of Relative Gene Expression

The relative expression of each target gene was calculated using the 2^(-ΔΔCt) method (24). First, the ΔCt for each sample was obtained by subtracting the Ct value of GAPDH from the Ct value of the target gene (ΔCt = Ct_target – Ct_GAPDH). Then, the ΔΔCt was calculated by subtracting the mean ΔCt of the control group (PBS-treated mice) from the ΔCt of each experimental group sample (ΔΔCt = ΔCt_experimental – ΔCt_control). Finally, the fold change in gene expression relative to the PBS control group was determined as 2^(-ΔΔCt). The PBS control group was arbitrarily set to a fold change of 1.0.

2.13. Statistical Analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). The normality of data distribution was evaluated using the Shapiro–Wilk test. As all datasets satisfied the assumption of normality, parametric statistical methods were subsequently employed. Differences among the four experimental groups were assessed using one-way analysis of variance (ANOVA). When a statistically significant overall ANOVA result was obtained ($p < 0.05$), Tukey's honest significant difference (HSD) post hoc test was performed to evaluate pairwise group comparisons. Statistical significance was defined at a two-tailed p-value threshold of less than 0.05. Levels of statistical significance were denoted as follows: $p < 0.05$, $p < 0.01$, and $p < 0.001$. For the sake of clarity

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and conciseness, the Results section reports only comparisons involving the PBS control group and the comparison between the free peptide and NLME groups, although all possible pairwise comparisons were performed during the statistical analysis.

3. Results

3.1. Bioinformatics Analysis Confirms High Antigenicity and Non-Toxicity of the Merged Peptide

Prior to synthesis, the merged multi-epitope peptide was extensively evaluated using in silico tools to predict its immunogenic potential and safety. The final sequence of 44 amino acids contained 6 high-affinity MHC class I epitopes (three from AFP, two from GPC3, and three from TERT) and 4 MHC class II epitopes. The VaxiJen v2.0 server predicted an antigenicity score of 0.78 (threshold > 0.4 indicating probable antigen), which falls in the high range. AllerTOP v2.0 classified the peptide as non-allergen, and ToxinPred analysis showed no toxicity-associated motifs. The I-TASSER-predicted three-dimensional structure revealed a flexible conformation with accessible epitope surfaces, suitable for processing by antigen-presenting cells. These in silico results collectively supported the experimental use of this peptide (**Supplementary 1**).

3.2. Nanoliposomes Exhibited Optimal Physicochemical Properties for Vaccine Delivery

Dynamic light scattering analysis of the NLME formulation revealed a unimodal size distribution with a mean hydrodynamic diameter of 105 ± 12 nm (Table 2). The size distribution histogram is presented in Figure 1A. The polydispersity index (PDI) was 0.21 ± 0.03, indicating a relatively homogeneous population with low aggregation tendency. The mean zeta potential was measured as +30.4 ± 2.1 mV (Figure 1B). This positive surface charge is desirable for electrostatic interaction with the negatively charged membranes of dendritic cells and macrophages, thereby enhancing cellular uptake and subsequent antigen presentation.

Transmission electron microscopy (TEM) of negatively stained nanoliposomes showed spherical, unilamellar vesicles with a dark electron-dense periphery and a lighter central core, consistent with the expected morphology of liposomes. The sizes observed by TEM were slightly smaller (approximately 90–100 nm) than those measured by DLS, which is expected due to dehydration during sample preparation and the fact that DLS measures the hydrodynamic diameter including the hydration layer. Peptide loading efficiency was remarkably high at 89.4% ± 3.2% (Table 2). This high encapsulation is attributed to the combined effects of the freeze-thaw cycling, which increases the internal aqueous volume, and the electrostatic attraction between the positively charged nanoliposome membrane and the peptide (which has an isoelectric point of approximately 9.5, making it positively charged at pH 7.4).

Table 2. Physicochemical characteristics of the NLME nanoliposomal vaccine (mean ± SD, n = 3 independent batches)

Parameter	Value
Mean hydrodynamic diameter (nm)	105 ± 12
Size range (nm)	60 – 180
Polydispersity index (PDI)	0.21 ± 0.03
Zeta potential (mV)	+30.4 ± 2.1
Peptide loading efficiency (%)	89.4 ± 3.2

3.3. Stability of NLME Formulation Was Maintained for 4 Weeks at 4°C

The stability study results are summarized in Table 3. When stored at 4°C, the NLME formulation showed minimal changes in mean diameter (from 105 nm to 108 nm after 28 days, within ±3% of initial) and PDI (remained below 0.25). The zeta potential decreased slightly from +30.4 mV to +27.8 mV after 4 weeks, but this difference was not statistically significant ($p > 0.05$). Peptide leakage (the percentage of initially encapsulated peptide that was found in the supernatant after ultracentrifugation) was only 4.2% at day 28. In contrast, when stored at 25°C, a significant increase in size was observed (from 105 nm to 142 nm by day 28, $p < 0.05$), accompanied by an increase in PDI to 0.38, indicating particle aggregation. Peptide leakage at 25°C reached 15.8% by day 28. Therefore, all subsequent experiments used freshly prepared NLME or formulations stored at 4°C for no more than one week.

Table 3. Stability parameters of NLME formulation over 4 weeks at 4°C (mean ± SD, n = 3)

Parameter	Day 0	Day 7	Day 14	Day 21	Day 28
Mean size (nm)	105 ± 12	106 ± 11	106 ± 13	107 ± 12	108 ± 12
PDI	0.21 ± 0.03	0.22 ± 0.02	0.23 ± 0.03	0.24 ± 0.02	0.24 ± 0.03
Zeta potential (mV)	+30.4 ± 2.1	+30.1 ± 2.0	+29.5 ± 1.9	+28.9 ± 2.2	+27.8 ± 2.3
Peptide leakage (%)	0	1.2 ± 0.3	2.1 ± 0.4	3.0 ± 0.5	4.2 ± 0.6

3.4. NLME Vaccine Significantly Upregulated Pro-Inflammatory Cytokine Gene Expression in the

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Spleen

The relative expression levels of five inflammatory cytokine genes (IFN-γ, TNF-α, IL-6, IL-1β, and IL-10) in the spleens of immunized mice are presented in Table 4 and graphically in Figure 2.

Table 4. Relative expression of inflammatory genes in the spleen of mice (fold change vs. PBS control, mean ± SD, n = 6)

Cytokine	PBS control	Empty liposome	Free peptide	NLME vaccine
IFN-γ	1.00 ± 0.08	1.12 ± 0.11	2.34 ± 0.25	5.67 ± 0.48††
TNF-α	1.00 ± 0.10	0.98 ± 0.09	1.89 ± 0.18	4.23 ± 0.39††
IL-6	1.00 ± 0.07	1.05 ± 0.08	1.45 ± 0.14	2.12 ± 0.20†
IL-1β	1.00 ± 0.09	1.08 ± 0.10	1.62 ± 0.15	2.98 ± 0.27††
IL-10	1.00 ± 0.06	0.95 ± 0.07	0.78 ± 0.09	0.42 ± 0.05††

- p < 0.05 vs. PBS control
- p < 0.01 vs. PBS control
- † p < 0.05 vs. free peptide
- †† p < 0.01 vs. free peptide

IFN-γ: The NLME vaccine group exhibited the highest upregulation of IFN-γ among all groups, with a 5.67-fold increase compared to the PBS control ($p < 0.01$). This was significantly higher than the free peptide group (2.34-fold, $p < 0.01$). The empty liposome group showed no significant difference from PBS ($p > 0.05$).

TNF-α: Similar to IFN-γ, TNF-α expression was markedly elevated in the NLME group (4.23-fold, $p < 0.01$ vs. PBS). The free peptide induced a modest but significant increase (1.89-fold, $p < 0.05$ vs. PBS). The difference between NLME and free peptide was statistically significant ($p < 0.01$).

IL-6: NLME vaccination resulted in a moderate but statistically significant increase in IL-6 expression (2.12-fold, $p < 0.05$ vs. PBS). Free peptide induced a 1.45-fold increase, which was not significantly different from PBS ($p > 0.05$). The NLME group was significantly higher than the free peptide group ($p < 0.05$).

IL-1β: NLME induced a 2.98-fold increase in IL-1β expression ($p < 0.01$ vs. PBS). Free peptide induced a 1.62-fold increase ($p < 0.05$ vs. PBS). NLME was superior to free peptide ($p < 0.01$).

IL-10: Notably, the immunosuppressive cytokine IL-10 was significantly downregulated in the NLME group (0.42-fold, $p < 0.01$ vs. PBS), indicating a reduction in regulatory T-cell activity. The free peptide group also showed a modest but significant downregulation (0.78-fold, $p < 0.05$ vs. PBS). The difference between NLME and free peptide was significant ($p < 0.01$).

4. Discussion

The present study successfully developed a nanoliposomal vaccine encapsulating a bioinformatically designed merged multi-epitope peptide derived from three distinct HCC-associated antigens—AFP, GPC3, and TERT—and demonstrated its ability to elicit a potent Th1-polarized inflammatory response within the spleens of immunized mice. The selection of these three antigens was based on several complementary considerations. AFP remains the most extensively investigated serological biomarker of HCC, and AFP-derived peptide vaccines have been evaluated in multiple clinical studies, where they have demonstrated the capacity to induce antigen-specific T-cell responses (7,25). However, the emergence of AFP-low-expressing or AFP-deficient tumor variants under immune selective pressure may compromise the long-term effectiveness of AFP-based monovalent vaccines (26). GPC3 serves as an important complementary target because it is expressed in the majority of AFP-negative HCC tumors and has been shown to induce robust cytotoxic T-lymphocyte (CTL) responses that are associated with improved survival outcomes in patients vaccinated with GPC3-derived peptides (8,27). TERT constitutes a broadly applicable tumor-associated antigen owing to its reactivation in more than 85% of human malignancies, including HCC, and its indispensable role in maintaining telomerase activity required for unrestricted cellular proliferation (9). The incorporation of all three antigens into a single merged peptide construct reduces the likelihood of immune escape resulting from antigen loss while simultaneously broadening the diversity of activated T-cell clonotypes (28).

The utilization of a merged (consecutive) multi-epitope peptide construct, rather than a simple combination of individual peptide components, confers several practical and immunological advantages. First, it ensures the simultaneous delivery of all epitopes to the same antigen-presenting cell (APC), thereby facilitating coordinated Th1 immune activation (29). Second, it streamlines manufacturing processes, quality assurance procedures, and

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regulatory evaluation relative to formulations comprising multiple independently synthesized peptides (30). Third, the incorporation of overlapping epitopes capable of presentation through both major histocompatibility complex (MHC) class I and class II pathways within a single linear sequence promotes functional cross-talk between $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, a process that is essential for the establishment of durable immunological memory (31). Bioinformatic analyses conducted in the present study confirmed the presence of multiple high-affinity binding motifs for both $H\text{-}2K^d$ (MHC class I) and $I\text{-}A^d$ (MHC class II) alleles, suggesting that the merged peptide is likely to undergo efficient processing and presentation through both antigen presentation pathways. The critical role of the delivery system is evident from the comparison between the free peptide and NLME vaccine groups. While the free peptide did induce significant but weak upregulation of IFN-γ (2.34-fold) and TNF-α (1.89-fold), these responses were substantially lower than those achieved with nanoliposomal encapsulation (5.67-fold and 4.23-fold, respectively). This finding aligns with the well-established concept that soluble peptides, even if highly immunogenic in silico, are poorly immunogenic in vivo due to rapid clearance by renal filtration, proteolytic degradation in serum, and inefficient uptake by dendritic cells (32). Nanoliposomes overcome these barriers by protecting the peptide from enzymatic degradation, prolonging its circulation time, and promoting targeted delivery to secondary lymphoid organs such as the spleen (33).

The nanoliposomes prepared in this study exhibited several characteristics that are considered optimal for vaccine delivery. The mean size of 105 nm falls within the ideal range of 80–120 nm reported for efficient lymphatic drainage and uptake by APCs (34). Particles smaller than 20 nm are rapidly cleared by the kidneys, while particles larger than 200 nm tend to remain at the injection site and are poorly transported to lymph nodes (35). The narrow size distribution (PDI = 0.21) indicates a homogeneous population, which is important for reproducibility and batch-to-batch consistency in vaccine manufacturing (36). The positive zeta potential of +30 mV is particularly noteworthy. Positively charged liposomes interact electrostatically with the negatively charged glycosaminoglycans on the surface of dendritic cells and macrophages (37). This interaction not only enhances cellular uptake but also triggers a "danger signal" that promotes DC maturation and upregulation of co-stimulatory molecules (CD80, CD86) via activation of the NF-κB pathway (38). In contrast, neutral or negatively charged liposomes show significantly lower uptake by APCs and weaker immunogenicity (39). The high peptide loading efficiency of 89.4% ensures that the majority of the antigen is delivered in encapsulated form, minimizing waste and reducing the required dose. The freeze-thaw cycling technique employed here is known to increase encapsulation efficiency by creating larger internal aqueous spaces due to ice crystal formation, which upon thawing collapses to form unilamellar vesicles with high entrapment of soluble cargos (40).

The stability findings demonstrating that NLME formulations remained physically and chemically stable for a minimum of four weeks at 4°C possess important implications for translational and clinical applications. Since many liposome-based vaccine formulations require maintenance of a cold-chain distribution system, the observed stability under refrigerated storage conditions, characterized by minimal particle size variation (increase <10%) and limited peptide leakage (<5% after four weeks), indicates that this formulation possesses favorable characteristics for future clinical development, provided that these results can be consistently reproduced under large-scale manufacturing conditions (41).

One of the most notable findings of the present study was the 5.67-fold elevation in splenic IFN-γ expression observed in mice immunized with the NLME vaccine relative to PBS-treated controls. IFN-γ is recognized as the hallmark cytokine of type 1 helper T (Th1) cells, cytotoxic $CD8^+$ T lymphocytes, and natural killer (NK) cells (42). Within the context of cancer immunotherapy, IFN-γ mediates a broad spectrum of antitumor activities. First, it enhances the expression of MHC class I molecules on tumor cells, thereby increasing their recognition by $CD8^+$ CTLs (43). Second, it promotes activation of the immunoproteasome, resulting in more efficient processing and presentation of tumor-associated antigens (44). Third, it suppresses angiogenesis through the inhibition of pro-angiogenic mediators, including vascular endothelial growth factor (VEGF) (45). Fourth, it facilitates the polarization of tumor-associated macrophages (TAMs) from the immunosuppressive M2 phenotype toward the pro-inflammatory M1 phenotype, thereby further reinforcing Th1-mediated immune responses (46).

Importantly, the magnitude of IFN-γ induction observed in the NLME group is comparable to, and in some cases exceeds, values reported for other nanoliposome-based cancer vaccine platforms. For instance, Liu et al. (2022) reported a 4.2-fold increase in splenic IFN-γ expression following administration of a liposomal multi-epitope melanoma vaccine (47). Likewise, Wang et al. (2021) documented a 3.5-fold increase in IFN-γ expression using an AFP-based nanovaccine in experimental HCC models (48). The superior induction of IFN-γ observed in the present

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study may be attributable to the synergistic incorporation of three highly immunogenic tumor-associated antigens together with the optimized design of the cationic liposomal delivery system. Importantly, the free peptide alone induced only a 2.34-fold increase, confirming that nanoliposomal encapsulation is not merely a passive carrier but an active adjuvant. This adjuvant effect likely involves several mechanisms: (i) protection from degradation, allowing more intact peptide to reach the spleen; (ii) enhanced DC uptake and cross-presentation; (iii) activation of the inflammasome pathway by the liposomal lipids themselves; and (iv) promotion of germinal center reactions in the splenic white pulp (49). It is also worth noting that IFN-γ can have dual roles in cancer—while it is predominantly anti-tumor, chronic exposure to high levels of IFN-γ can induce PD-L1 upregulation on tumor cells and T-cell exhaustion (50). However, in the context of vaccination in tumor-free mice as performed here, the high IFN-γ response is interpreted as a favorable predictor of anti-tumor efficacy.

The NLME vaccine also induced significant upregulation of TNF-α (4.23-fold) and IL-1β (2.98-fold). TNF-α, originally identified for its ability to induce hemorrhagic necrosis of tumors (51), acts synergistically with IFN-γ to enhance CTL activity. TNF-α activates caspases in cancer cells, inducing apoptosis through both the extrinsic (death receptor) and intrinsic (mitochondrial) pathways (52). Additionally, TNF-α enhances the adhesion of immune cells to tumor endothelium by upregulating adhesion molecules such as ICAM-1 and VCAM-1, facilitating CTL infiltration into the tumor microenvironment (53). However, it is important to acknowledge that TNF-α can also promote tumor progression under certain circumstances, particularly when present chronically at low levels, by activating NF-κB and inducing anti-apoptotic proteins in cancer cells (54). In the setting of a therapeutic vaccine, the transient and localized production of TNF-α in lymphoid organs is thought to be beneficial rather than harmful.

IL-1β is a potent pro-inflammatory cytokine produced by activated macrophages and DCs following activation of the NLRP3 inflammasome (55). Although IL-1β has historically been associated with fever and acute inflammation, recent studies have revealed its critical role in promoting Th17 differentiation and enhancing the expansion of memory CD8+ T cells (56). Moreover, IL-1β signaling in DCs enhances their capacity to prime naive CD8+ T cells, which is essential for generating effective anti-tumor immunity (57). It is noteworthy that the empty liposome group did not significantly increase IL-1β compared to PBS, indicating that the liposomes themselves did not trigger strong inflammasome activation. The increase observed in the NLME group is therefore likely due to the peptide cargo itself, possibly because the multi-epitope peptide contains motifs that are recognized by pathogen recognition receptors (PRRs) or because the positively charged liposome-peptide complex is particularly efficient at inducing phagosomal rupture and cytosolic release of antigen, a known trigger for NLRP3 activation (58).

Unlike the strong upregulation of IFN-γ and TNF-α, IL-6 showed a more modest but significant increase (2.12-fold) in the NLME group. IL-6 is a pleiotropic cytokine with context-dependent roles in cancer (59). On one hand, IL-6 can promote the differentiation of naive CD4+ T cells into Th17 cells, which produce IL-17 and recruit neutrophils to the tumor site, sometimes leading to anti-tumor effects (60). IL-6 also enhances the survival of activated T cells by upregulating the anti-apoptotic protein Bcl-2 (61). On the other hand, chronic elevation of IL-6 is associated with HCC progression, metastasis, and poor prognosis, primarily through activation of the JAK/STAT3 signaling pathway in hepatocytes and HCC cells (62). The 2.12-fold increase observed in our study is relatively modest compared to the dramatic elevation seen in chronic inflammatory conditions (often > 10-fold). Furthermore, this increase was accompanied by high IFN-γ and low IL-10, indicating that the overall balance is pro-inflammatory rather than tumor-promoting. In the context of a vaccine administered subcutaneously, the transient production of IL-6 in the spleen likely contributes to the initial activation of T cells without causing systemic IL-6-mediated pathology (63).

One of the most encouraging findings was the significant downregulation of IL-10 in the NLME group (0.42-fold). IL-10 is the primary immunosuppressive cytokine produced by regulatory T cells (Tregs), M2 macrophages, and certain subsets of B cells (64). In the tumor microenvironment, IL-10 inhibits the activation of DCs, suppresses the production of pro-inflammatory cytokines by T cells, and directly impairs the cytotoxic function of CD8+ T cells (65). High serum levels of IL-10 in HCC patients correlate with advanced stage, poor response to therapy, and shorter overall survival (66). Therefore, a vaccine that not only induces pro-inflammatory cytokines but also actively suppresses IL-10 production is highly desirable. The mechanism by which NLME downregulates IL-10 is likely indirect: the strong Th1 response (high IFN-γ) inhibits the differentiation of naive CD4+ T cells into FoxP3+ inducible Tregs (iTregs), which requires TGF-β and IL-2 but is antagonized by IFN-γ (67). Additionally, IFN-γ promotes the development of Th1 cells that

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produce no IL-10 or, in some cases, produce IL-10 as a late feedback mechanism to limit immunopathology (68). However, the net effect in our study at day 42 (14 days post-final boost) is clearly a suppression of IL-10, which bodes well for the vaccine's ability to prevent immune tolerance.

Several previous studies have explored nanoliposome-based vaccines for HCC using single antigens. For example, Zhang et al. (2019) formulated liposomal AFP peptide (AFP158-166) with CpG adjuvant and reported a 3.1-fold increase in splenic IFN-γ and a 2.8-fold increase in TNF-α (69). Our NLME vaccine induced higher responses (5.67-fold and 4.23-fold, respectively), likely due to the multi-epitope design and the absence of a separate adjuvant (the cationic liposome itself serves as an adjuvant). Another study by Li et al. (2020) used GPC3 peptide alone in anionic liposomes and reported a 2.5-fold increase in IFN-γ (70). Again, our results surpass those findings. A notable study by Zhao et al. (2021) combined AFP, GPC3, and TERT peptides as a mixture in Freund's adjuvant, achieving a 4.1-fold increase in IFN-γ (71). Although this is higher than our free peptide (2.34-fold), it is still lower than our NLME vaccine (5.67-fold), indicating that the nanoliposomal platform is superior to Freund's adjuvant, which is not clinically applicable due to severe local inflammatory reactions (72). To our knowledge, this is the first study to report such high IFN-γ induction using a merged multi-epitope peptide encapsulated in cationic nanoliposomes without additional adjuvants.

The findings of this study have several translational implications. First, the splenic inflammatory gene expression profile—high IFN-γ, TNF-α, moderate IL-6/IL-1β, and low IL-10—provides a strong rationale for evaluating the NLME vaccine in orthotopic HCC mouse models (e.g., Hepa1-6 subcutaneous or intrahepatic injection). If these Th1 responses translate into tumor growth inhibition and prolonged survival, the vaccine could be advanced to toxicology studies in non-human primates. Second, the stability data at 4°C suggest that this vaccine can be stored and distributed using existing cold chain infrastructure, which is feasible for clinical use. Third, the use of a merged peptide rather than multiple separate peptides simplifies Good Manufacturing Practice (GMP) production and reduces costs. Fourth, the absence of any additional adjuvant (e.g., CpG, poly(I:C), or alum) reduces potential toxicity and regulatory hurdles because liposomal lipids are generally recognized as safe (GRAS) by the FDA (73).

However, several limitations must be acknowledged. First, this study was conducted in healthy mice without any pre-existing HCC. It is possible that the presence of a tumor, which often establishes an immunosuppressive microenvironment, may blunt the vaccine-induced inflammatory response. Second, we did not directly assess the anti-tumor efficacy or T-cell cytotoxicity (e.g., chromium release assay or ELISpot). Cytokine gene expression in the spleen is an indirect measure of T-cell activation. Third, we did not evaluate the frequency of antigen-specific T cells using tetramer staining or intracellular cytokine staining following re-stimulation. Fourth, the study only evaluated responses up to day 42; the durability of the responses (e.g., at 6 months or 1 year) remains unknown. Fifth, the immunized mice were female only. Sex differences in vaccine responses are well-documented, with females often mounting stronger responses (74). Future studies should include both sexes.

Therefore, future work should include: (1) Therapeutic efficacy studies in subcutaneous and orthotopic HCC models, comparing NLME vaccine to controls; (2) Measurement of antigen-specific CTL activity; (3) Long-term memory evaluation by rechallenging mice with tumor cells after 6 months; (4) Assessment of the vaccine in aged mice (which have declining immune function) to model the typical HCC patient population (median age > 65 years); (5) Biodistribution studies to confirm that the nanoliposomes indeed target the spleen and lymph nodes; (6) Evaluation of combination therapy with immune checkpoint inhibitors (anti-PD-1, anti-CTLA-4) to potentially overcome resistance; (7) Safety and toxicology studies in rats or rabbits.

Why did the NLME vaccine induce such strong responses in the spleen? The spleen is uniquely positioned to capture blood-borne antigens due to its highly vascularized structure and the presence of specialized marginal zone macrophages and metallophilic macrophages that express high levels of scavenger receptors (75). Subcutaneously administered nanoparticles enter the lymphatic circulation and drain to the inguinal and axillary lymph nodes, but a significant fraction eventually reaches the bloodstream and is filtered by the spleen (76). The optimal size of 105 nm allows for efficient passage through the lymphatic vessels (which have inter-endothelial gaps of up to 200 nm) and subsequent entry into the splenic white pulp through the marginal sinus (77). The positive zeta potential of +30 mV further enhances retention in the spleen because negatively charged endothelial cells in the marginal zone facilitate electrostatic trapping of cationic particles (78). Once in the splenic white pulp, the nanoliposomes are taken up by CD8+ conventional dendritic cells (cDC1s) and CD4+ cDC2s, which then migrate to the T-cell zones and prime naive T cells (79). The merged multi-epitope peptide, being processed and presented on both MHC class I and II, activates both CD4+ and CD8+ T cells, and the

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activated T cells produce IFN-γ and TNF-α, completing the feedback loop.

5. Conclusion

In conclusion, the present study successfully developed a cationic nanoliposomal vaccine encapsulating a bioinformatically designed merged multi-epitope peptide derived from AFP, GPC3, and TERT for the immunotherapeutic targeting of hepatocellular carcinoma. The resulting NLME formulation exhibited favorable physicochemical characteristics, including a mean particle diameter of 105 nm, a positive zeta potential of +30.4 mV, a high peptide encapsulation efficiency of 89.4%, and satisfactory stability for at least four weeks under refrigerated storage conditions (4°C). Immunization of BALB/c mice with the NLME vaccine elicited a pronounced Th1-polarized inflammatory response within the spleen, as evidenced by substantial upregulation of IFN-γ (5.67-fold) and TNF-α (4.23-fold), moderate increases in IL-1β (2.98-fold) and IL-6 (2.12-fold) expression, and marked downregulation of the immunosuppressive cytokine IL-10 (0.42-fold). Notably, these immunological effects were significantly greater than those induced by the corresponding free (non-encapsulated) peptide formulation, underscoring the critical roles of the nanoliposomal platform in both antigen delivery and immune potentiation. The observed cytokine profile, characterized by a strongly pro-inflammatory and Th1-skewed immune milieu accompanied by suppression of regulatory signaling pathways, provides compelling immunological evidence supporting the potential of the NLME vaccine as a promising candidate for HCC immunotherapy. Nevertheless, further investigations employing orthotopic tumor models and comprehensive toxicity assessments in large-animal systems are required to facilitate the translation of these findings toward clinical application.

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Ethical Considerations and Compliance with Ethical Guidelines

The research protocol was reviewed and approved by the Ethics Committee of the Department of Biology, Faculty of Biosciences, North Tehran Branch Islamic Azad University Tehran, Iran (Approval No: IR.IAU.TNB.REC.1403.143). All methods for the animal experiments are approved by ARRIVE guidelines (https://arriveguidelines.org). All humans and/or human data were in compliance with the Helsinki Declaration (https://www.wma.net/policies-post/wma-declaration-of-helsinki/). This study adhered to the Declaration of Helsinki. Also, all human procedures were approved by the Ethics Committee of the North Tehran Branch of Islamic Azad University (IR.IAU.TNB.REC.1403.142).

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Conflict of interest

There is no conflict of interest.

AI Using Declaration

During the preparation of this manuscript, the authors used DeepSeek-V3 (accessed on May 3, 2026) solely for the purpose of final language editing, grammatical refinement, and checking for potential unintentional plagiarism.The tool was not used to generate research data, analyze results, or draw scientific conclusions. The authors have reviewed and edited the final output and take full responsibility for the content of the manuscript.

Author's contributions

Conception and design: M.H, A.J, M.S, Provision of study: M.H, E.S, A.J, and M.E, Collection, and assembly of data: M.H, A.J, and M.S, Data analysis and interpretation: M.H, E.S, A.J, and M.E, Manuscript writing and final approval of manuscript: all authors.

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