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Triple Tandem Mimotope Peptide of Epidermal Growth Factor Receptor Displaying on the Surface of M13 Phage Induces Anti-Tumor Response in Mice Tumor Model

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Introduction: Epidermal growth factor receptor (EGFR) has been shown to play a critical role in tumor cell growth and its overexpression has been observed in many epithelial tumors. In the field of cancer vaccine research, displaying the peptide mimotope on the surface of phage particles has shown promising results.
Materials and Methods: In this study using m13-PVIII phage display system, two constructions were prepared: triple tandem repeat of EGFR mimotpe displaying particles (3M) and single EGFR mimotope displaying phage particle (1M). To investigate the anti-tumor properties of phage vaccine, C57BL/6 mice Lewis lung carcinoma xenograft model was established and treated with 3M phage vaccine, 1M phage vaccine and control agents.
Results: Immunization of mice with these phage-based vaccines showed strong immune response against phage-mimotope. 3M phage vaccine showed more potency against tumor in comparison with control groups. Also the survival time was extended in phage vaccine treated tumor-bearing mice compared with untreated mice.
Conclusions: Our findings suggest that mimotope-displaying phage vaccine can induce specific antibodies with antitumoral activity, which its potential as a candidate vaccine for EGFR-specific cancer immunotherapy needs to be more investigated in future studies.

Keywords: EGFR; Immunotherapy; Mimotope; Phage vaccine

1. Background

Epidermal growth factor receptor (EGFR) has been shown to play a critical role in tumor cell growth and its over expression has been reported in a significant percentage of human tumors such as those of breast, lung, prostate, head and neck, colorectal, pancreatic, bladder, vulva and ovarian tumors (1, 2). This over expression has been correlated with progression and poor prognosis of disease (3). EGFR or HER1 is one of the four members of erbB cell membrane receptor family. The binding of ligands, such as epidermal growth factor (EGF) or transforming growth factor alfa (TGF-α) to the extracellular domain of the receptor results in the activation of the EGFR and its downstream cell signaling molecules leading ultimately to tumour cell proliferation, reduced apoptosis, increased angiogenesis and increased migration and invasion (4).

Today cancer immunotherapy is a promising approach against cancer (5-7). Among a variety of immunotherapy approaches such as using DNA plasmids (8), dendritic cells (9) or xenogeneic endothelial cells (10) that have been used in cancer therapy, bacteriophage display represent a powerful system for the delivery of the desirable peptides to the activation of the immune system and its anti tumor properties. Filamentous bacteriophages (m13/fd) were first used for displaying peptides (11) and are still a predominant phage strain. While size restriction in displays have been reported in some studies, filamentous bacteriophages are efficiently able to induce both humoral and cellular immune responses making them an attractive tool as an antigen delivery system in vaccinations (12).

Mimotopes, mimetics of epitopes, are developed using combinatorial peptide libraries and can

efficiently be displayed on the surface of phage particles and induce immune response. The sequence of natural epitopes and their mimotopes can be different. Mimotopes are usually more potent and they can be selected from peptide libraries and tested for their potency to induce immune response (13, 14). In our laboratory, EGFR mimotope was isolated from a peptide library, using anti EGFR monoclonal antibody ICR62 by phage display technology. In this study two m13 phage particles as a vaccine displaying three tandem repeats of EGFR mimotope and the other one a single EGFR mimotope at N-terminal of major coat protein VIII were made and their prophylactic and therapeutic effects of both particles were investigated in mice tumor model.

2. Materials and Methods

2.1. Construction of EGFR mimotope-PVIII displaying m13 phage particles

Two constructions containing EGFR mimotope, were prepared. EGFR mimotope sequence (5′-CAGCATTATAACATTGTGAACACCCAGAGCCGCGTG-3′) was cloned between SfiI and NotI restriction sites in PAK-8 phagemid vector (kindly provided by Dr. Klimka, University of Cologne, Cologne, Germany) and in the other construction a triple tandem repeat of mimotope was cloned of the mentioned phagemid vector (Figure 1). Chemical competent cells of the TG1 *E. coli* host were prepared and transformed by EGFR mimotope containing phagemid vector using heat-shock method. Bacterial strain were infected by KO7 helper phages to produce PVIII-mimotope displaying m13 phage particles. Then the phage particles were concentrated and tittered according to Wan *et al*. (15).

2.2. Anti-mimotope ELISA

To evaluate the reaction of displayed peptide mimotope on the suface of phage particles an ELISA was carried out. The wells of maxisorb plate (Nunc, USA) were coated with polyclonal anti peptide antibody (prepared in our laboratory by immunization of rabbits using peptide mimotope). After blocking (skim milk 5% in PBS for 2 h at 37ºC) the wells were washed three times with PBST (PBS, pH 7.2 containing 0.1% Tween-20). Then 1M-phage and 3M-phage (10^8 phage particles in 100 µL of PBS) were added and incubated 1 h at room temperature. After three times washing with PBST, HRP-conjugated anti-m13 phage monoclonal antibody (GE Healthcare, USA) were added in 1:8000 dilution in 1% BSA in PBST for 1 h at RT. The wells were washed three times with PBST and then the color development reaction was performed using TMB (Sigma, USA) substrate. Reactions were stopped with 50 µL 2 M HCl and absorption was measured at 450 nm using ELISA plate reader (Awareness Technology, Inc., USA). Irrelevant peptide displaying phage particles and irrelevant polyclonal antibodies were used as negative peptide and antibody controls, respectively.

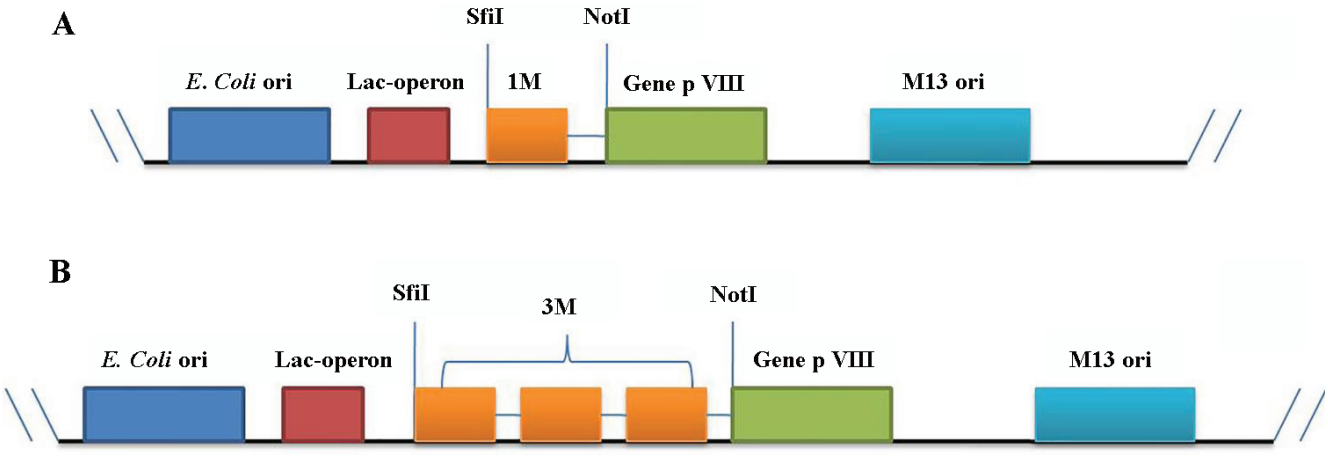

Figure 1. Schematic representation of the phagemid constructs containing EGFR mimotope. (A) PAK-8 phagmid vector containing single copy of EGFR mimotope (1M). (B) PAK-8 phagmid vector which in, a triple tandem repeat of EGFR mimotope is cloned. In both constructs the mimotope was cloned between *SfiI* and *NotI* restriction sites

2.3. Preventive and therapeutic studies on mouse Lewis lung carcinoma tumor model

2.3.1. Animals

Forty-eight 6-8 week-old inbred female C57BL/6 mice were obtained from Pasteur Institute of Iran (Tehran, Iran). All the animal studies were performed in accordance with international guidelines and approved by our institutional ethics board.

For preventive studies, twenty-four C57BL/6 mice were divided into four groups (n = 6/each group). Mice were subcutaneously immunized with the phage-based mimotope vaccines at the titer of 10^{13} phage particles/ml during one month. The experimental groups were injected with 100 µL of: 1M-phage (1M-P group) (P stands for Prophylactic), 3M-phage (3M-P group), empty helper phage (H-P group) and PBS buffer (P-P group) respectively. On the 7th day of the 31 days of immunization, each mouse was inoculated with 10^6 Lewis lung carcinoma cells (LL/2).

LL/2 cells are tumor cell strain and have been reported as an important tumor model for cancer studies (16-18). Tumor volume was determined by the following formula: tumor volume (mm^3) =1/2A (mm) $\times B^2$ (mm^2), where 'A' denotes the largest dimension of the tumor and 'B' represents the smallest dimension (19).

To evaluate the therapeutic effect of phage-mimotope particles against established tumor, twenty-four C57BL/6 mice were divided into four groups (n=6/each) and received: 1M-phage (1M-T group) (T stands for Therapeutic), 3M-phage (3M-T group), empty helper phage (H-T group) or PBS (P-T group), 7 days after challenging the mice with 10^6 LL/2 tumor cells. Tumor growth was monitored by measuring subcutaneous tumors two to three times a week with a digital caliper and its volume determined by the above mentioned formula.

2.3.2. Cytokine analysis

Blood samples were collected from tail vein and prepared serum samples were stored at -20ºC. Serum levels for IL-4 and IFN-γ were measured using sandwich-based PEPROTECH Murine cytokine ELISA kits (PEPROTECH, USA) according to the manufacturer's instruction. In brief, 100 µL of capture antibodies were added to a 96-well plate. The plates washed twice (PBS, pH 7.2 containing 0.1% Tween-20) and after 2 h of blocking (1% BSA in PBS), 100 µL of standards and serum (diluted 2-fold) were added to the wells, for 2 h at room temperature. Wells were washed three times, followed by the addition of 100 µL of detection antibodies. The plate was incubated as previously mentioned. After three washes, 100 µL of Avidin-HRP were added to each well and incubated at room temperature for 30 min. Wells were washed three times and then substrate was added to each well. The plate was read using ELISA plate reader at 450 nm.

2.3.3. Flow Cytometry analysis

LL/2 cells were detached from the tissue culture flask with trypsin and EDTA (Sigma Aldrich), washed with PBS and centrifuged at 2000 g in 1.5 ml microfuge tubes twice. Approximately 3×10^5 cells were placed on ice and incubated with 100 µL immunized mice serum antibody in for 60 min. They were subsequently washed twice with PBS at 4ºC. The secondary layer was a goat anti-mouse Ig-FITC polyclonal antibody (Razi Biotech, Iran) which was applied for 60 min. Cells were then washed three times with PBS at 4ºC, and analyzed with BD FACSCanto II flow cytometer (BD Biosciences, USA) equipped with a Argon blue laser (488-nm). Irrelevant mouse polyclonal antibodies were used as isotype control antibodies to assess the background staining. After gating on forward and side-scatter parameters, at least 10,000 gated events were analyzed using FlowJo software. Flow cytometry analyses were repeated twice.

2.3.4. Statistical analysis

Values were expressed as means ± SEM. Comparative analyses were performed using the Student's t test and the results of tumor growth were evaluated by one-way ANOVA followed by a Dunnett's multiple comparison test. Results were all considered to be statistically significant when $P < 0.05$. Statistical analysis and drawing figures were made by Graph Pad Prism version 5.04 software and Microsoft Office Excel.

3. Results

3.1. Construction of PVIII-EGFR mimotope displaying M13 phage particles

Tandem repeat of EGFR mimotope (containing GGGGS linker between repeats) was synthesized (Gen Script, USA) and cloned between *SfiI* and *NotI* restriction sites of PAK8 phagemid vector. Also another construct of PAK8 vector, containing a single copy of mimotope was prepared. The successful cloning was verified by sequencing (MWG, Germany).

3.2. Anti-mimotope ELISA

The results confirmed the displaying of peptide mimotope on the surface of phage particles and also the specific reaction of peptide with anti-peptide antibody. Irrelevant-peptide displaying phage particles and also irrelevant antibody were used as controls (Figure 2).

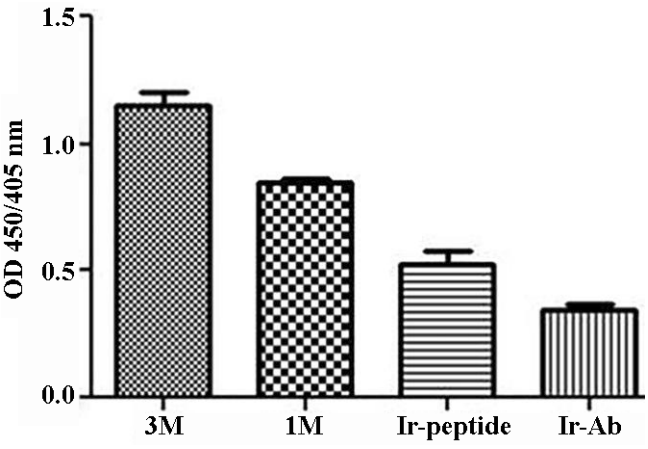

Figure 2. Anti-peptide ELISA representing the specific reaction of peptide-mimotope on the surface of phage particle toward anti-peptide antibody. The specific reaction of peptide-mimotope on the surface of phage particle toward anti-peptide antibody were assessed. 3M-mimotope displaying phage particles (3M), 1M-mimotope displaying phage particles (1M), irrelevant peptide displaying phage particles (Ir-peptide) and irrelevant polyclonal antibody (Ir-Ab). The experiments repeated twice and data shown are the mean of duplicates

3.3. The protective effects of the phage vaccines

To investigate the protective properties, the mice were immunized subcutaneously with the phage vaccines on days 0, 10, 17, 24 and 31 and then challenged with LL/2 tumor cells. It was observed that the rate of tumor growth was slower in phage vaccine injected groups than the control groups and a significant difference in tumor growth rate was observed between mice in 3M-P and P-P group ($P<0.05$) (Figure 3A). Additionally, the survival length was extended in 3M-P group compared with control group mice. On day 38, all mice in the P-P group died out, whereas in the 3M-P group mice, just one mice died at the same day. Furthermore, 3M-P obviously improved the median survival of the mice bearing tumors for 11 days in comparison with P-P control group. The survival rate of 3M-P group on day 50 was 16.6%, whereas control groups merely had a 16.6% survival rate on day 38 (Figure 3B).

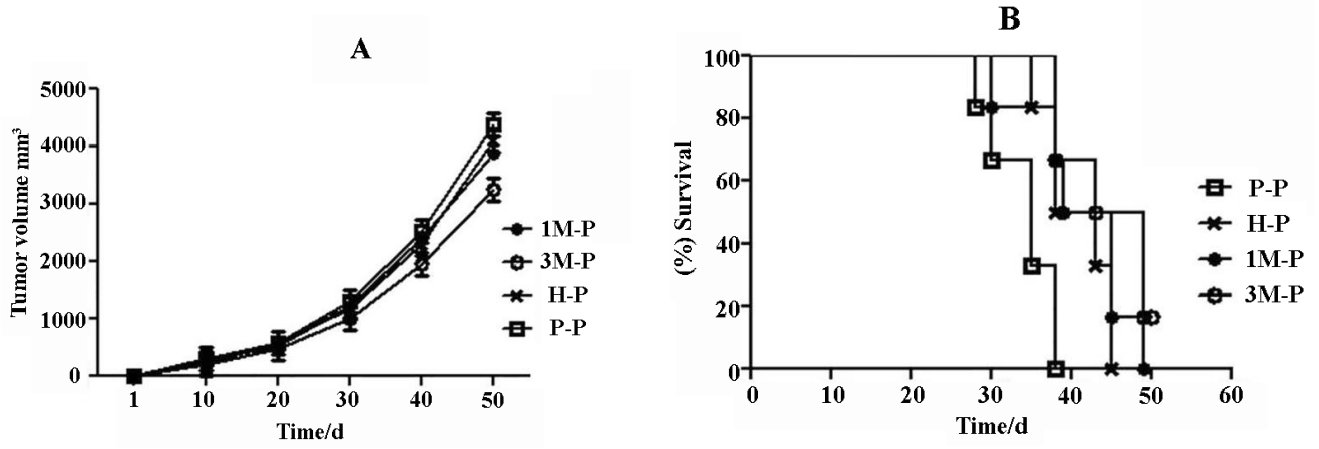

Figure 3. The protective effects of phage vaccines. Mice (6 per group) were immunized with 100 mL of mimotope-phage (10^{13} particles/mL). The groups were presented as: 3M phage treated group (3M-P)(o); 1M phage treated group (1M-P)(•); blank m13 helper phage receiving group (H-P)(×) and PBS buffer group (P-P)(□). Starting to measure at day 10th after 1×10^6, Lewis lung carcinoma cells (LL/2) were subcutaneously injected to mice. Within phage vaccine treated groups, the results showed significant difference between tumor growth of 3M-P and P-P control group (A). Survival curves of LL/2 engrafted mice during the observation. The survival length in tumor-bearing mice was extended in the 3M-P group compared with the control groups using Log-Rank (Mantel-Cox) test ($P < 0.05$) (B)

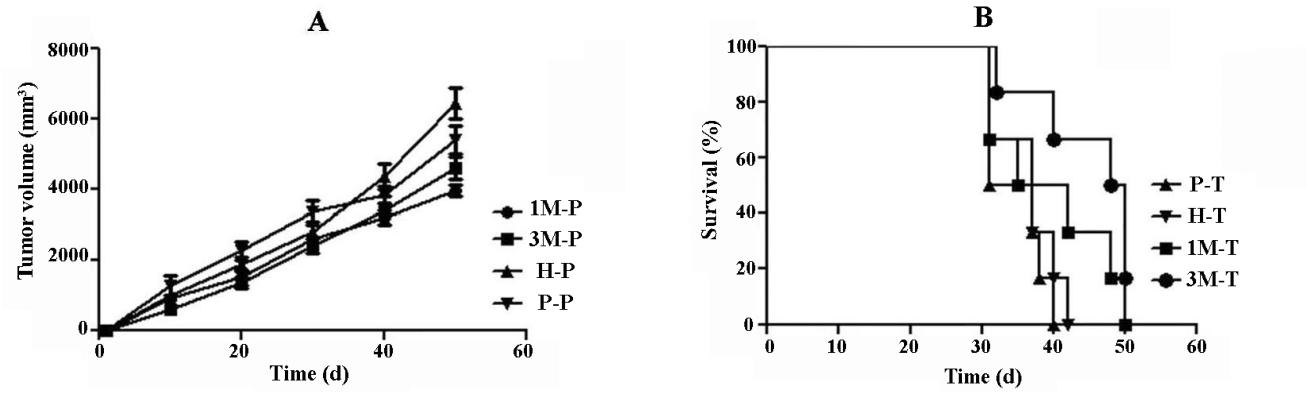

Figure 4. The therapeutic effects of the phage vaccines. Mice (6 per group) were immunized with 100 µL of mimotope-phage (10^{13} particles/ml), 7 days after challenging them subcutaneously with 1×10^{6}, Lewis lung carcinoma cells (LL/2). The phage vaccines were 1M-phage group (1M-T)(■) and 3M-phage group (3M-T)(●). Blank m13 helper phage (H-T)(▼) and PBS buffer (P-T)(▲) were used as control. The results showed a significant difference between tumor growth rate of 3M-T group with H-T and P-T control groups (A). Mouse survival duration. The survival length of mice in 3M-T group was showed obviously extended compared with the mice in control groups, using Log-Rank (Mantel-Cox) test (P = 0.02) (B)

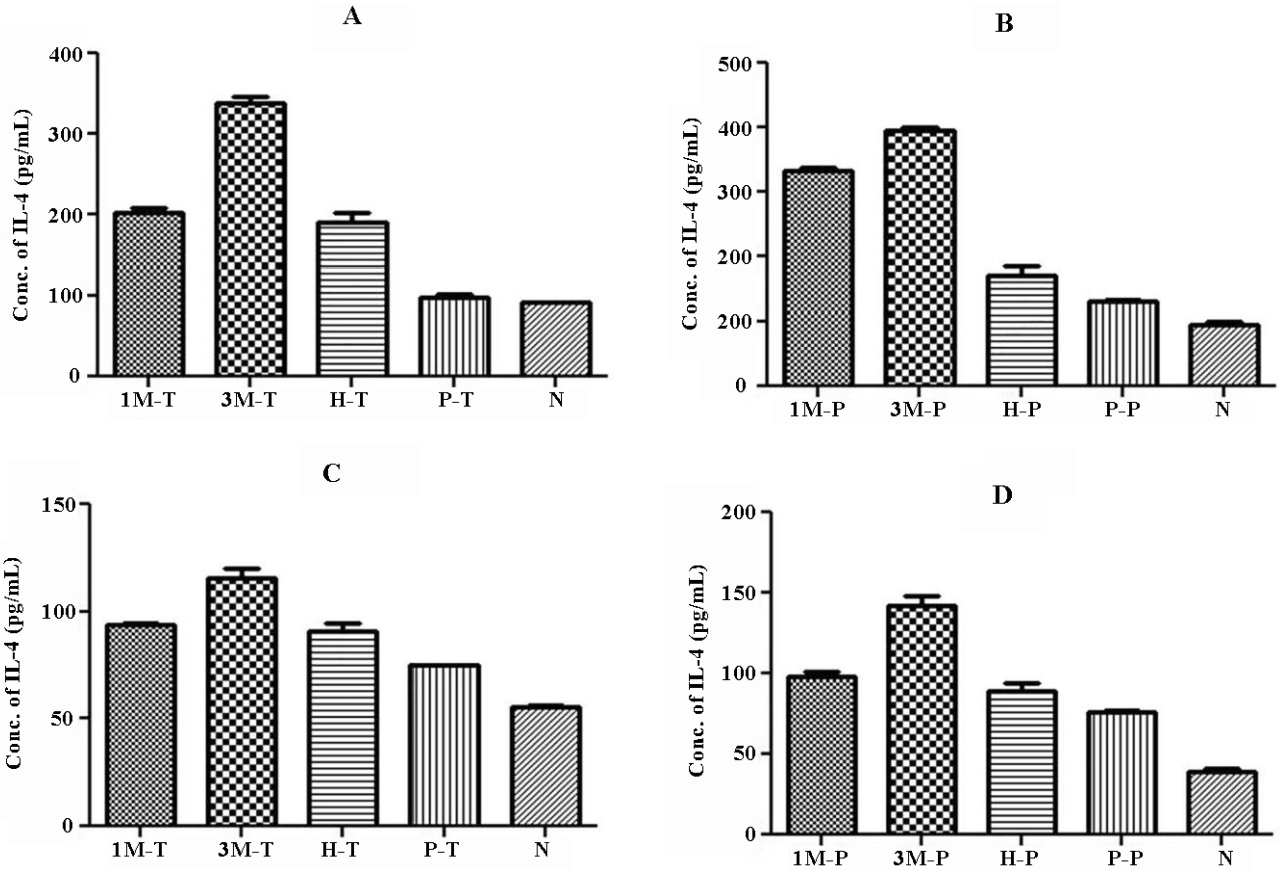

Figure 5. The mice in therapeutic and prophylactic groups were bled after immunization with mimotope-phage particles and serum levels of IL-4 and IFNγ (pg/ml) were measured. Concentration of IL-4 in therapeutic group mice (A), Concentration of IL-4 in prophylactic group mice (B), Concentration of IFNγ in therapeutic group mice (C) and concentration of IFNγ in prophylactic group (D). (1M-T: therapeutic group immunized with 1M-phage particles, 3M-T: therapeutic group immunized with 3M-phage particles, H-T: therapeutic group immunized with blank helper phage particles, P-T: therapeutic group received PBS, N: normal mouse serum. 1M-P: prophylactic group immunized with 1M-phage particles, 3M-P: prophylactic group immunized with 3M-phage particles, H-P: prophylactic group immunized with blank helper phage particles, P-P: prophylactic group received PBS). Bars represent mean values ±SD

3.4. Therapeutic activity assay of phage vaccines

To determine the therapeutic antitumor activity of recombinant phages, first the mice were challenged with LL/2 cells and after 7 days, they were injected subcutaneously with phage vaccines for four weeks. The results showed a significant difference between tumor growth rate of 3M-T group and the control groups ($P < 0.05$), but it was not observed any significant difference between P-T and H-T groups (Figure 4A). On day 40, all the mice in P-T group died. On the same day, among the 3M-phage treated group (3M-T) mouse survival was significantly affected by the treatment, respectively. Median survival of 3M-T group was observed 15 days more than of P-T control group.

On day 50 the survival rate was 16.6% in the experimental groups (3M-T and 1M-T), whereas for the P-T control group and for the H-T control group the survival rate was 16.6% on day 39 and day 41, respectively (Figure 4B).

3.5. Cytokine analysis

Sandwich-based ELISA assays were performed to detect the concentration of serum IL-4 and IFN-γ cytokines. The mice immunized with phage vaccines showed the higher level of cytokines and so increased immunity, compared with the mice in the control groups (Figure 5).

3.6. Flow cytometry analysis

To verify the raised humoral immunity against vaccine and also interaction of mice polyclonal antibodies with LL/2 cancerous cells, flow cytometry analysis was performed. The results showed that the humoral response was raised aginst phage-mimotope and also showed that the potency of tandem mimotope-displaying phage particles (3M) to raise antibody against EGFR, is more than single-mimotope displaying particles (1M). The specific reaction of mice antibodies toward EGFR were also observed, from the negative results of control groups (Figure 6).

4. Discussion

The biology of Epidermal Growth Factor Receptor (EGFR) as the major receptor to response to the growth activity of EGF has made it suitable as a potential target for anti-cancer therapy. To the present, the EGFR-targeted anti-cancer therapies include anti-EGFR antibodies (20), tyrosine kinase (TK) inhibitors (21), antisense oligonucleotides (22), and EGFR-directed mimotope vaccines (23, 24).

One of the main strategies in cancer vaccine design is the administration of immuno dominant epitope to elicit the immune response (25). Today in many studies mimotopes are considered as alternatives to natural epitopes for the immune therapy of cancer (13). Riemer *et al*. isolated the EGFR mimotope using cetuximab (anti-EGFR monoclonal antibody) and their immunization results showed that the raised anti-mimotope antibodies could efficiently recognize EGFR and also inhibited the growth of EGFR expressing cells to

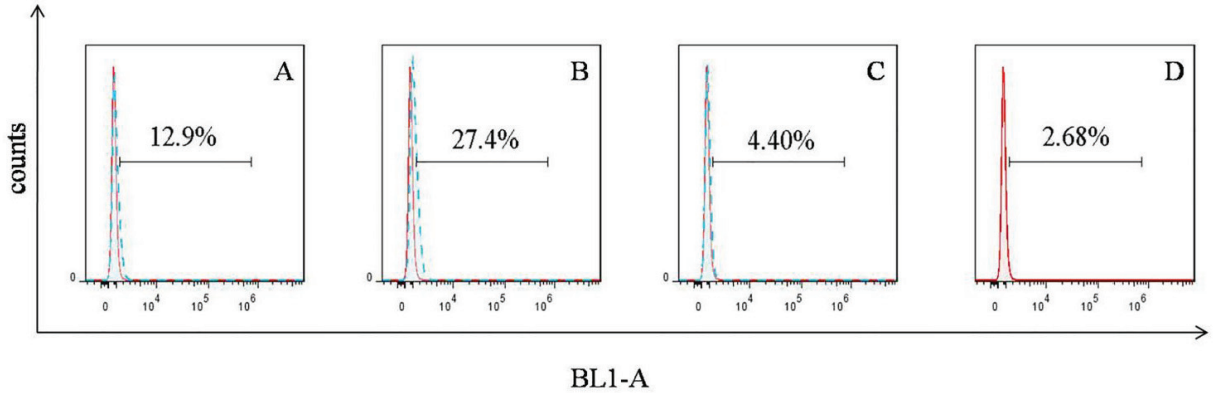

Figure 6. Flow cytometry analysis. Investigation of induced humoral response and specific reactivity of mice serum toward LL/2 cells. LL/2 cells were incubated with 100 µl of mice polyclonal antibodies and then analyzed by flow cytometry for specific reaction of mice antibodies with LL/2 cells. 1M-phage mice serum (A), 3M-phage mice serum (B), iso control (C) and negative control (D). The red histograms indicate background staining correction and the blue-dashed histograms show the the specific staining percentages of the cells by antibodies

a similar extend as cetuximab (26). Mimotopes are usually peptide molecule, they structurally mimic epitopes (14) have advantages that have made their application more feasible than original antigens or their epitopes in cancer immunotherapy studies: 1-easy production because of their short linear sequence instead of the original epitopes that in most cases are conformational. 2-Mimicking non-protein antigens such as carbohydrates. 3-The ability to find mimotopes for unknown antigens, since there is no need to have information about the sequence of original antigen in the process of discovering mimotopes (14, 27, 28).

In our experiment, we constructed m13 phage vaccines displaying EGFR mimotope on the major coat protein-VIII surface to induce the humoral immune response to treat the tumor-bearing mice.

Phage display is a powerful technique which is used to display exogenous peptides on their surface as a fusion to phage proteins. Filamentous bacteriophages may represent a powerful antigen delivery system that can be utilized to design and develop new safe and inexpensive cancer vaccines. Peptides displayed on the surface of filamentous bacteriophages are able to induce humoral as well as cell-mediated immune responses. Bacteriophages are easy to prepare in large quantities at a very low cost and are very stable (29). Over the last several years filamentous phage based vaccines displaying immunogenic peptides demonstrated a great deal of promise in very diverse areas, including infectious diseases, cancer and neurological disorders (30).

In this study we used the strategy of expressing mimotope on the surface of phage particles as low molecular weight substitutes of the natural EGFR for active immunization that could potentially elicit specific antibodies resulting into the induction of long-lasting humoral immune responses. Several studies have shown the effect of *in vivo* response induced by phage-displayed mimotopes (31-33).

To investigate the anti-tumor effects of our vaccines, C57BL/6 mice tumor model was established by inoculation of LL/2 Lewis lung carcinoma cell line. This cell line has been widely used to establish the model of EGFR-positive tumor *in vivo*. Dong et al. by inoculation of LL/2 cells into C57BL/6 mice, investigated the anti-tumor properties of T7 phage-based EGFR vaccine (7). Lu *et al*. also by establishment of xenogeneic EGFR tumor model using LL/2 cells, studied the efficiency of DNA-based EGFR vaccine in mice (34). In our study in order to show the prophylactic effects of the phage vaccine, mice were immunized for one month with the phage vaccines before they were inoculated with the LL/2 tumor cells. A significant tumor volume difference was not observed between the 3M-P and 1M-P groups, while the results showed a significant difference between 3M-P and P-P control group. At the end of the experiment a significant extended survival rate was observed in 3M-P group mice compared with the control group. While all the mice in the P-P control group died out just one of the mice in the 3M-P group had died, at the end of the experiment 16.6% survival rate was observed in 3M-P group. This result indicates that phage-based mimotope vaccine may have the potent *in vivo* antitumor activity.

In therapeutic groups no significant difference was observed between the tumor growth rate of 3M-T group and 1M-T group. But tumor growth rate of 3M-T group (not 1M-T group) showed significant difference in comparison with P-T and H-T control groups. At the end of the experiment 16.6% improved length of survival rate was observed in 3M-T group compared with H-T and P-T control groups.

Although in both prophylactic and therapeutic groups no significant difference was observed between potency of 3M and 1M phage vaccines, the tumor growth study showed that 3M-phage vaccine was more potent in comparison with 1M-phage vaccine in both groups. Also the extended survival rate in animals treated with 3M-phage vaccines could confirm the efficiency of this vaccine compared with 1M-phage vaccine. Ren et al. also produced T4-mVEGFR2 phage vaccine and their *in vivo* immunotherapy experiments showed extended survival in LL/2 inoculated mice (6).

To study the polarization of immune system in the mice injected by phage-based mimotope vaccines, levels of IL-4 and IFN-γ cytokines were measured in the serum of immunized mice. In our immunization groups we observed a Th2 polarized humoral response. Wan et al in immunization of mice with tumor displaying phage particles resulted in Th1-dominated immune response characterized by phage particle-specific secretion of

IFN-γ but not IL-4(15). Thomas et al also immunized the mice with λ-phage particles and demonstrated a Th1 response but also a Th2 antibody response (35). But Ren et al anti-angiogenic T4-mVEGFR2 vaccine results showed antitumor activity of T4-mVEGFR2 and production of VEGF-specific antibodies (6).

In this experiment in order to find the raised antibodies reacting towards the natural antigen on the surface of tumor cells, a flow cytometry-based assay was carried out. We assayed LL/2 cells with mouse anti-phage-mimotope serum labeled with goat FITC-conjugated anti-mouse antibody. The results showed that 3M-phage vaccines raised the anti EGFR antibodies in a more efficient manner than 1M-phage particles. This data may support the better results of 3M-phage vaccine anti-tumor properties in comparison with 1M-phage vaccine.

Our data also showed that the peptide mimotope displayed on the surface of phage particle mimics structural features of EGFR, and as immunogen induced antibodies in animals that cross-reacts with EGFR.

In conclusion our results show that immunization with mimotope-displaying phage particles can trigger an effective humoral immune response in mice and produce a relative protection against EGFR positive tumor. This data may be helpful for further clinical cancer immunotherapy research.

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Authors Contribution

Study concept,design and supervision: Mohammad Javad Rasaee, Masoud Javanmardi. Acquisition, Analysis and interpretation of data: Mohammad Javad Rasaee, Masoud Javanmardi and Mohammad Ghaem maghami. Drafting of the manuscript: Masoud Javanmardi. Critical revision of the manuscript for important intellectual content: Mohammad Javad Rasaee and Helmout Modjtahedi. Statistical analysis: Masoud Javanmardi, Majid Asadi-Ghalehni, Mohammad Ghaem maghami. Administrative, technical, and material support: Masoud Javanmardi, Mohammad Javad Rasaee and Helmout Modjtahedi.

Financial Disclosure

The authors have no financial conflicts of interest.

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