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Construction of a Recombinant Phage-vaccine Capable of Reducing the Growth Rate of an Established LL2 Tumor Model

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ABSTRACT

Over expression of the epidermal growth factor receptor (EGFR) in many human epithelial tumors has been correlated with disease progression and poor prognosis. EGFR-inhibiting immunotherapy has already been introduced in cancer therapy. Peptide displaying phage particles in eukaryotic hosts can behave as antigen carriers, able to activate the innate immune system and to elicit adaptive immunity.

Herein, the M13-pAK8-VIII phagemid plasmid was engineered to contain the sequences for an EGFR mimotope along with the L2 extracellular domain of EGFR (EM-L2) which would produce the final peptide-phage vaccine. The prophylactic and therapeutic effects of this novel vaccine were evaluated on the Lewis lung carcinoma induced mouse (C57/BL6) model.

The recombinant peptide was confirmed to be displayed on the surface of M13 phage as an extension for phage's PVIII protein. Immunization of mice with peptide-phage vaccine resulted in antibody production against EM-L2 and significant reduction of tumor growth rate by nearly 25 percent.

In conclusion, EM-L2 displaying phage particles could be deemed as an encouraging strategy in contemporary cancer immunotherapy.

Key words: Cancer; Epidermal growth factor receptor; Immunotherapy; Peptide vaccine

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INTRODUCTION

The EGFR is a 1210 amino acids protein consists of extracellular ligand-binding (ECD), transmembrane and intracellular domains with tyrosine kinase activity for signal transduction. This receptor is involved in various neoplastic processes. It is reported to be overexpressed or mutated in numerous human tumors including breast, lung, prostate and head and neck tumors. Regardless of the EGFR wide expression in various organisms, it is a compelling target for cancer therapy.[1,2] Extracellular domain of the EGFR consists of 4 domains: L1, S1, L2 and S2.[3] It is known that L1 and L2 domains are devoted to ligand binding.[4] However, domain L2 appears to contribute most of the binding energy and is of more pivotal role in Epidermal Growth Factor (EGF) binding.[5] Moreover, Ramires et al showed that mice immunization with the extracellular domain of murine EGFR (mEGFR-ECD) (emulsified in adjuvants) can solve the tolerance problem against self EGFR. They have shown that the immunization with extracellular domain can induce an efficient immune response capable of exerting antitumor effects.[6]

Various methods of cancer therapy have been developed based on EGFR targeting using monoclonal antibodies or small molecule suppressors.[7,8] However, these therapies could be associated with limitations like issues of biocompatibility, low stability, pharmacokinetics properties, attaining consistent dose, high prices and stimulation of host immune system.[9,10] Active immunization against cancer antigens is an alternative strategy in cancer immunotherapy. In this regard, the use of phages to display various proteins on their surface become to be a promising method.[11] Previously we have mapped ICR-62 (an EGFR monoclonal antibody) and reported an EGFR mimotope, short peptide molecules mimicking epitope structure.[12] It was reported that rabbit IGs against this mimotope could successfully react with the EGFR protein.[12] Thereafter, this mimotope was displayed on M13 phage surface as a fusion with pVIII coat protein. Prophylactic and therapeutic potentials of this bacteriophage-based mimotope vaccine proved the antitumor activities of the mimotope. The results showed that immunization of mice with these phage-based vaccines could be exploited in cancer immunotherapy.[13,14] Phages could easily be produced in high titers, their production is cost effective and they eliminate the need for an adjuvant.[15] It have been demonstrated that EGFR extracellular domain could be contemplated as an amenable adjuvant and its corresponding mimotopes delivered by an appropriate carrier are immunogenic enough to be used for cancer therapy.[6,14,16,17]

In the present study a combination of phage vaccination and EGFR based peptide active immunotherapy was considered as a complementary approach for treatment of $EGFR^+$ tumors. To this end, the DNA sequence encoding the EM.L2 recombinant peptide was optimized for expression in *E.coli* and was cloned into the M13-pAK8-VIII phagemide vector; the EM.L2 peptide was displayed on the M13 phage surface as a fusion protein with pVIII. The peptide specific antibody response was evaluated against EM.L2 peptide displaying phage and purified EM-L2 peptide. Ultimately, using ectopic Lewis lung carcinoma animal model prophylactic and therapeutic potentials of this new recombinant peptide-phage vaccine were evaluated.

MATERIALS AND METHODS

Construction of M13-pAK8-VIII-EM.L2 Phagemid

DNA sequence of EM.L2 peptide was obtained and codon optimized according to the *E. coli* codon preference (Table 1). The DNA fragment was chemically synthesized (444bp) and inserted into the M13-pAK8-VIII (Biomatik, USA/Canada) phagemid using Sfi1/Not1 restriction sites to construct the "M13-pAK8-VIII-EM.L2" phagemid plasmid.

Amplification and Titration of Recombinant Phage particles

Transformation of the *E. coli TG1* cells by the constructed M13-pAK8-VIII-EM.L2 plasmide and their superinfection by M13KO7 helper phage was used to produce EM.L2 displaying m13 phage particles. The method developed by Javanmardi et al was used to obtain these pahge particle.[18] Then, the phage particles were concentrated and tittered (for the number of their genome equivalents) according to the method developed by Wu et al[19] Briefly, after 2 h incubation on ice, the constructed phage particles were span down at 14000 rpm. Phage pellet was re-suspended in 2 mL of PBS and centrifuged at 4000 rpm to remove bacterial cell derbies.

Then, the phage particles re-precipitated by adding

Table 1. The amino acids and DNA sequences of L2 extracellular domain of EGFR (EM-L2) peptide

	Name	Sequence
1	Mimotope	QHYNIVNTQSRV
2	L2 Domain of EGFR	***CTAISGDLHILPVAFKGDSFTRTPPLDPRELEILKTVKEITGFLLIQAWPDNWTDLHAFENLEII RGRTKQHGQFSLAVVGLNITSLGLRSLKEISDGDVIISGNRNLCYANTINWKKLF***
3	EM-L2 Peptide Sequence	NH2-HHHHHH**QHYNIVNTQSRV**GGGGS***CTAISGDLHILPVAFKGDSFTRTPPLDPR ELEILKTVKEITGFLLIQAWPDNWTDLHAFENLEIIRGRTKQHGQFSLAVVGLNITSLGLRSLK EISDGDVIISGNRNLCYANTINWKKLF***GGGGS-COOH
4	Optimized DNA sequence of EM-L2 Peptide	CATCATCATCATCATCATCAGCACTATAACATCGTGAATACCCAGTCTCGCGTGGCGGTGG CGGCAGTTGTACGGCAATCAGCGGTGATCTGCATATTCTGCCGG TCGCATTTAAAGGCGATAGCTTTACCCGTACCCCGCCGCTGGACCCGCGTGAC TGGAAATTCTGAAAACCGTGAAAGAAATTACGGGTTTTCTGCTGATCCAGGCGTGGCCGG ATAACTGGACCGACCTGCATGCCTTCGAAAATCTGGAAATTATCCGTGGCCGCACGAAAC AGCACGGTCAATTTTCCCTGGCGGTGGTTGGCCTGAACT TACCAGCCTGGGTCTGCGTTCTCTGAAAGAAATCAGTGATGGCGACGTTATTATCAGCGGT AATCGCAATCTGTGCTACGCAAATACGATCAACTGGAAAAAACTGTTCGGTGGTGGTGGC AGCGC

The mimotope sequence is in bold and underlined, while the L2 domain is in bold and italicized. Using G4S linker, the 3 peptides are fused. National Center for Biotechnology Information (NCBI) Reference Sequence of epidermal growth factor receptor (EGFR) protein is: NP_997538.1

100 mL of PEG/NaCl solution, and re-suspended again in 2 mL PBS. Ultimately, phages were tittered using serial dilution method.

Phage Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot Analyses for the Evaluation of Displayed Peptide

1 µg of ICR62 (anti EGFR), ICR61 (anti EGFR), rabbit Immunoglobulin G (IgGs) against mimotope[12] and 5µg of anti-progesterone antibody (Ab) (as control) in 100 µL of Na2CO3/NaHCO3 buffer (0.1 M, pH 8.6) were used to coat the 96-well ELISA plates (NUNC, Maxisorb). The coated ELISA plates were incubated overnight at 4^{o} C. Thereafter, they were washed two times with TBST (50 mM Tris/HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.4) and blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature (RT). After discarding the blocking buffer and washing two times with TBST, 10^{12} plaque-forming units (PFU) of EM-L2 displaying phages (in 100 µL blocking buffer) were added to each well and incubated for 1 h at room temperature. Plates were washed three times with TBST before the addition of 100 µL horseradish peroxidase (HRP) conjugated anti-M13 (Pharmacia) (1:10000 diluted in TBST (v/v)) and incubated at 37^{o} C for 1 h. After washing for three times with TBST, 50 µL of TMB ELISA substrate solution (Sigma-Aldrich) was added and incubated for 15 minutes at RT. Reactions were stopped by 50 µL of 2M HCL. Microplate ELISA reader was used to read the absorbance values at 450 nm wavelength. The western blot was performed according to the method employed by Dong et al[10] using the ant-his-tag poly clonal antibody (HRP conjugated) (abcam ab1187) to detect the displayed peptide and the phage displaying β2m peptide as control. Briefly, the samples were electrophoresis on a 12% (v/v) SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was washed three times with TBST buffer. Then, the membrane was blocked with 5% (v/v) non-fat dry milk in PBS buffer for 90 minutes. Thereafter, the membrane was washed twice for 10 minutes each time with PBS buffer and incubated with HRP-conjugated anti His tag antibody (diluted 1:3000, v/v) (abcam) for 1 h at RT. The membrane was washed three times with TBST buffer for 10 minutes and ultimately was analyzed using the DAB substrate.

Immunization of Mice

4 groups of 4 BALB/c female mice, 6 weeks old (Pasteur institute of Iran), were obtained from Pasteur

Institute of Iran (Tehran, Iran). The first group (group EM.L2) was immunized subcutaneously (S.C.) by approximately 10^{12} PFU of EM.L2 displaying phages. The second group (group C) was immunized with 10^{12} PFU of beta2-microglobulin (beta2m) displaying phages dissolved in 150 µL PBS. The third group (L2pep) immunized by the EM.L2 peptide (100 ugr). The forth group (group PBS) received 150 µL PBS. The injections were carried out S.C., 4 times with 7-day intervals. The mice were bled 3 days after the last injection and before establishing the tumor model. Using 1:500, 1:2000 and 1:4000 dilutions of mice serum (serum/PBS, v/v), an ELISA test performed as described above. In each ELISA test the coated antigens were the antigens which were used for the immunization. This means that the immunized serums were tested with their own immunization antigens. The studies which involved animal use were approved by the Animal Care and Use Committee (ACUC) of Tehran University of Medical Sciences. Then, the EM-L2 peptide was expressed in *E. coli* BL21 (DE3) pLysS.[20]

Peptide ELISA of Mice Serum against EM.L2 Peptide

An ELISA test was designed to analyze whether the raised antibodies are capable of interacting with the peptide. The same procedure of ELISA test was employed to perform the ELISA of mice serum against EM.L2 peptide. The ELISA plate Wells were coated with 1 µg of purified EM.L2, while mice serum was added into the wells at 2 dilutions (1:1000 and 1:4000, serum/PBS, v/v) after the blocking. Finally, 100 µL of 1/10000 diluted HRP conjugated anti-mouse antibody in PBS (1:10000, v/v) (Pharmacia) was added to the wells and OD values were determined at 450 nm.

Cell Culture and Tumor Model Establishment

Lewis Lung carcinoma (LL/2) cells (National cell bank of Iran) were cultured in modified eagle media (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) at 37ºC in an atmosphere containing 5% (v/v) CO_2.[21]. At confluency of 90%, cells were trypsinized (Biosera, 1X, France) and washed by sterilized PBS. Cells were counted and re-suspended in DMEM media. To establish the tumor mass, a week after the last booster injection, approximately 10^6 cells in 100 µL DMEM media were injected S.C. to the right flank of the immunized C57BL6/J mice.

Investigation of Anti-Tumor Effects of Phage Vaccine in Mice

Twenty-four, six weeks old C57BL6/J mice (Pasture, Iran) were used to conduct the experiment. To evaluate the prophylactic effects, 12 mice were primarily immunized using the same regimen employed in section 2.4. A week after the last immunization, the mice were challenged S.C. with 10^6 live LL/2 cells. For the therapeutic studies, mice were first challenged S.C. in the right flank with 10^6 live LL/2 cells. When the tumors were palpable, the mice divided into 3 groups of 4 mice (t-EM-L2, t-C and t-PBS). Once again, the immunization regimen of section 2.4 was employed to immunize the mice with palpable tumors. The growth rate of both prophylactic and therapeutic groups was measured three times a week using digital caliper. Tumor sizes were expressed as the product of: $V=4/3\pi (R_1)^2(R_2)$, (where R_1 equals to largest diameter and R_2 equals to smallest diameter).

Statistical Analysis

Data were expressed as SEM. Comparative analyses were performed using the Student's T Test and the results of tumor volume were appraised by one-way ANOVA followed by Dunnett's multiple comparison tests. Results were all considered to be statistically significant when $p<0.05$. Statistical analysis and drawing of figures were made by Microsoft Office Excel.

RESULTS

Assessment of Displayed Peptide on Phage Surface with ELISA

The linear diagram of EM.L2 peptide is depicted in Figure 1. The obtained results have indicated that the phage particles were able to express the recombinant EM.L2 peptide on their surfaces. The observed 45% of OD difference between the wells assayed by specific and nonspecific antibodies could be a fair indication of displayed peptide Figure 2. The performed western blot results reveals that the recombinant peptide is expressed and displayed as a fusion for the phage PVIII protein Figure 2.

Mouse Immunization Assay

To evaluate the immunization of BALB/c mice, 3 different dilutions of mice serum (obtained by standard eye-bleeding) (serum/PBS, v/v) were prepared for

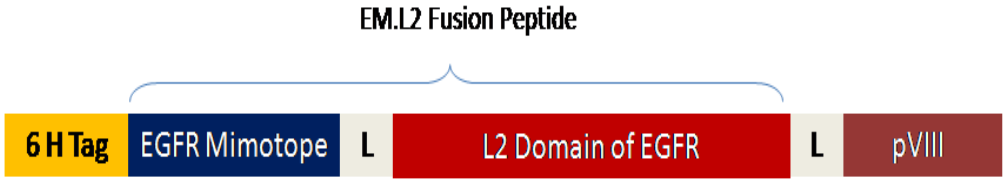

Figure 1. Linear diagram of EM.L2 peptide. The details are shown in the picture.

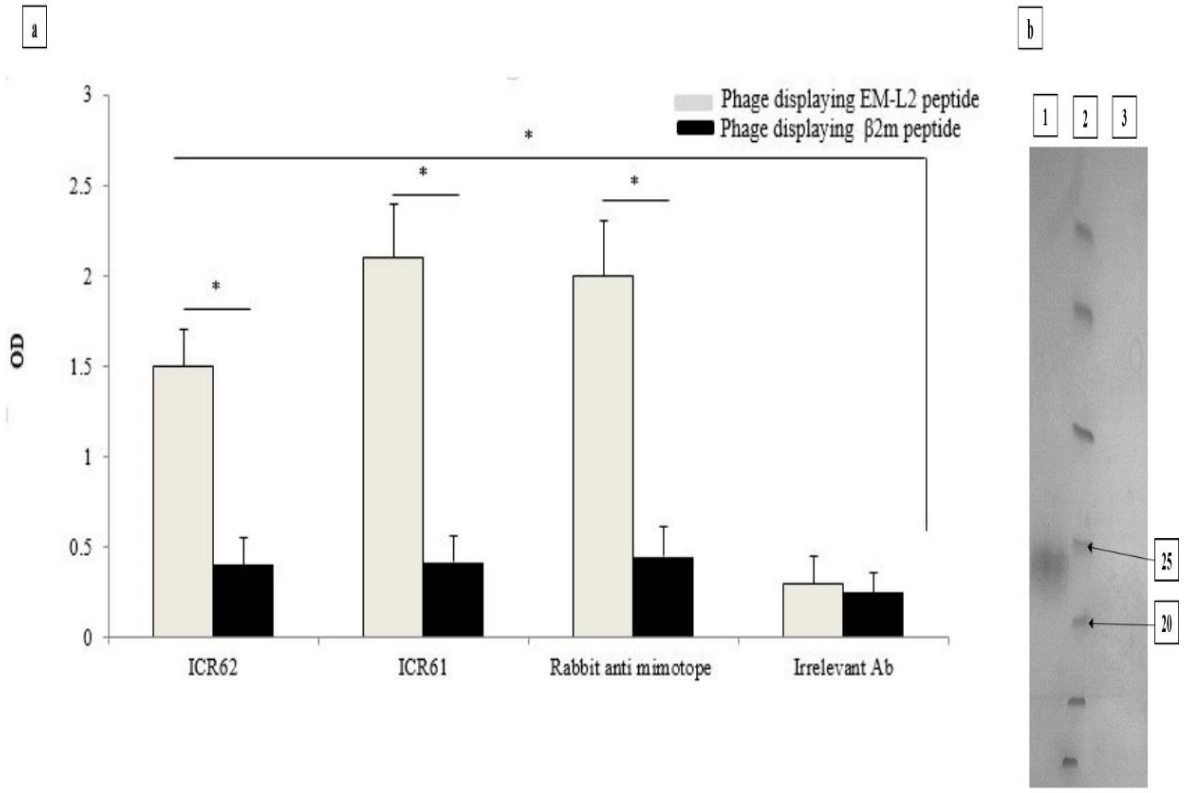

Figure 2. (a) ELISA results for EM-L2 peptide display on the surface of phage particles. ICR61 and ICR62 monoclonal antibodies and rabbit IgGs were against the mimotope, while anti-progesterone antibody was the irrelevant antibody as control. Beta2m displaying phage particles were used as control. Each bar represents the mean ±SEM (* p<0.05). (b) The western blot results. The existing of a 23 kDa protein band confirms the expression the fusion peptide (lane 1 is the phage displaying EM-L2 peptide, lane 1 is the protein ladder and lane 1 is the phage displaying β2m peptide).

phage ELISA (the serums were pooled together). The ELISA results (Figure 3) indicated that the performed immunizations have elicited high titers of anti-phage antibody compared to the control (immunized with PBS) group.

Evaluation of Mouse Sera against EM.L2 Recombinant Peptide

ELISA assay was performed to compare the affinity of serum antibodies of BALB/c mice groups towards the EM.L2 peptide. The results indicated that immunization of mice with phages displaying EM-L2 peptide resulted in successful antibody production against EM-L2. Two dilutions of sera were produced for the ELISA test confirmed that the produced polyclonal antibodies are capable of detecting displayed peptide. Further details are shown in Figure 4.

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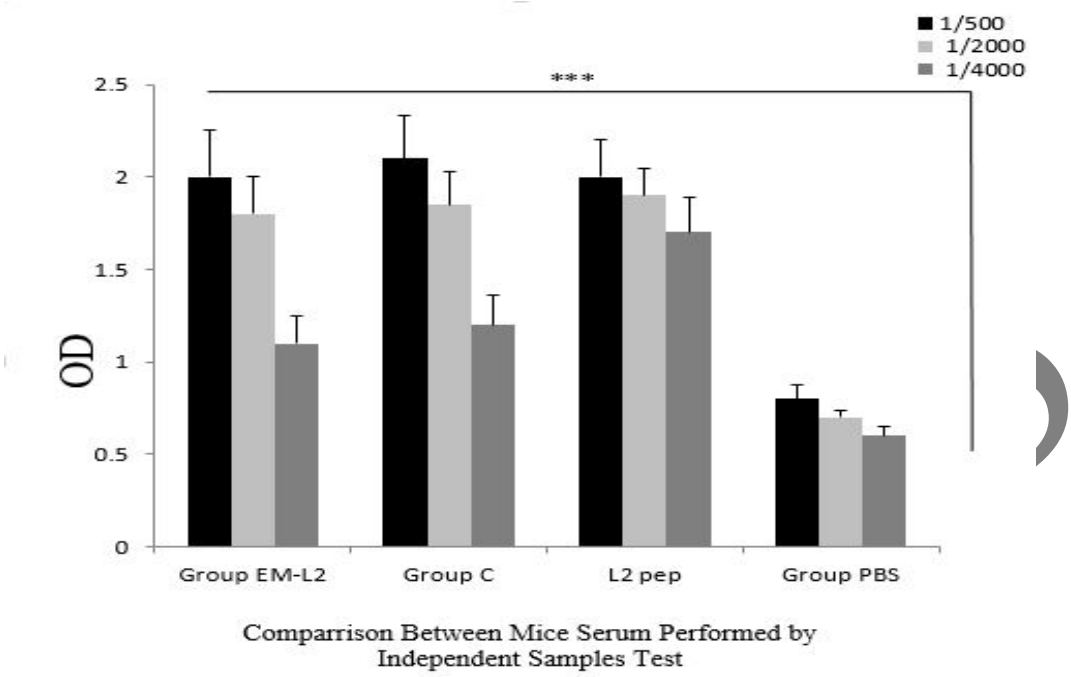

Figure 3. Mice immunization assay. Peptide displaying phage particles were coated in the wells .The Group EM-L: Mice pooled serum immunized by EM-L2 displaying phage particles. Group C: Mice pooled serum immunized by β2m displaying phage particles. Group L2 pep: Mice pooled serum immunized by L2 peptide. Group PBS: The pooled serum of non-immunized mice used as control. Each bar represents the mean ±SEM (* *p* <0.05).**

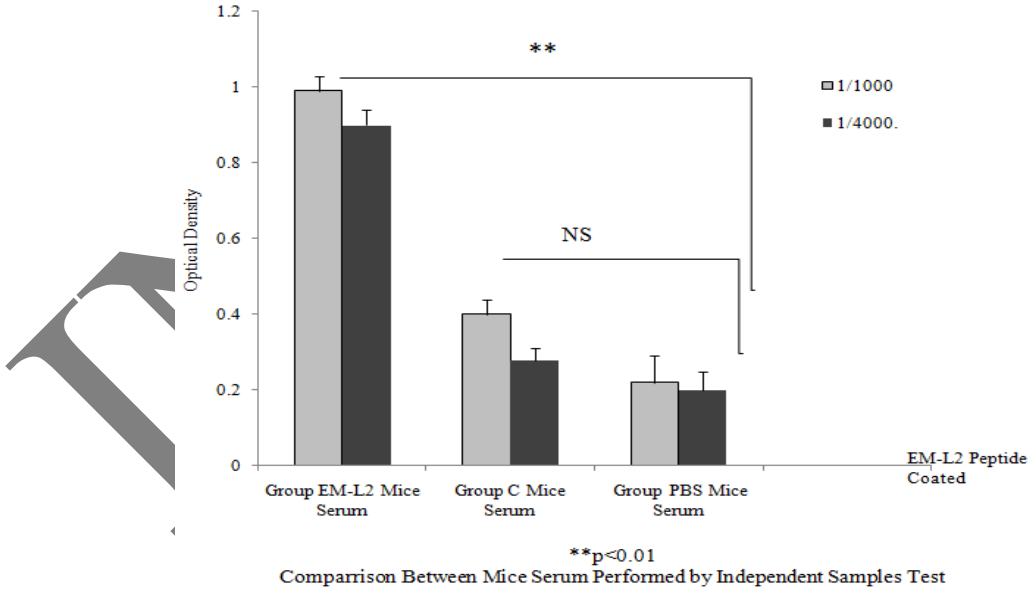

Figure 4. Affinity assessment of BALB/c mice sera against EM-L2 peptide. The peptide was coated and 2 dilutions (1/1000 & 1/4000) of sera were used for ELISA test. The results showed that mice sera immunized by EM-L2 displaying phage particles can significantly react against the peptide. Mice serums immunized by β2m displaying phage particles and pre-bleeding serum were used as controls. The group EM-L: Mice pooled serum immunized by EM-L2 displaying phage particles. Group C: Mice pooled serum immunized by β2m displaying phage particles. The pooled serum of non-immunized mice used as control. Each bar represents the mean ±SEM.

Prophylactic Properties of the Phage Vaccine

To evaluate the prophylactic effects, phage immunized mice were challenged S.C. by approximately 10^6 LL/2 cells. The results showed that the recombinant phage vaccine can reduce tumor growth in immunized mice. Significant differences in the tumor growth rate were observed between the PBS and the EM-L2 groups (p<0.05), while no significant difference between C and EM-L2 and between C and PBS groups were observed (Figure 5).

Therapeutic Properties of the Phage Vaccine

The mice challenged with EM-L2 phage vaccine and control phage have showed reduced the tumor growth in comparison to the group challenged with PBS. The results showed a significant difference between tumor growth rate of t-EM-L2 group and the control groups (p=0.011). However, no significant differences between control phage and PBS group were observed (Figure 6). Some pictures of the established tumors are presented in Figure 6.

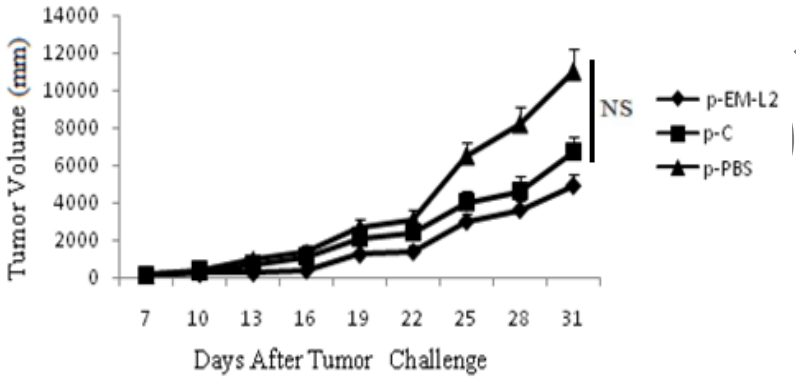

Figure 5. Prophylactic properties of EM-L2 peptide phage-based vaccine; p-EM-L2: mice group immunized using EM-L2 peptide phage-based vaccine, p-C: mice immunized with β2m displaying phage, p-PBS: mice immunized with Phosphate buffered saline (PBS). Once the tumors were palpable their dimensions was measured for four weeks. Significant difference was observed between p-EM-L2 and p-PBS groups. Not significant (NS) differences in tumor growth rate between the p-C and p- PBS groups were observed. Each bar represents the mean ±SEM (* p<0.05).

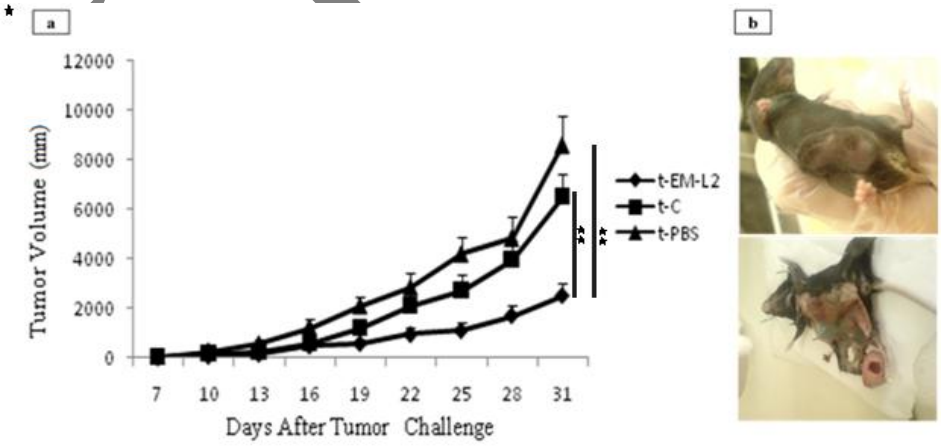

Figure 6. (a) Therapeutic properties of EM-L2 peptide phage-based vaccine; t-EM-L2: mice group injected by EM-L2 displaying phage vaccine, t-C: mice group injected by β2m displaying phage, t-PBS: mice group injected by PBS. Significant difference in tumor growth rate was seen between t-EM-L2 and control groups. There was no significant difference in tumor growth rate between the t-C and t-PBS groups. Each bar represents the mean ±SEM (p<0.05). (b) The pictures of the formed tumor before and after dissection.**

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DISCUSSION

In the present study, peptide vaccination and anti-cancer properties of phage particles were combined to construct a novel vaccine. We designed a recombinant peptide composed of the previously reported mimotope and L2 extracellular domain of the EGFR (EM-L2).[20] Since, in silico methods have been widely used to understand the mechanism and structure of various proteins[22-28] and vaccine design,[29-31] we have used this approach for our antigen design. It was anticipated that such a peptide could react with anti EGFR and anti mimotope antibodies. Therefore, EM-L2 peptide could behave as a more efficient vaccine for $EGFR^+$ tumors compared to mimotope by itself. To construct the recombinant phage vaccine, the whole EM-L2 peptide was displayed on M13 phage as a fusion to pVIII protein. The observed 45 % of OD difference (against anti EGFR antibodies) along with the results obtained from western blot analyses confirmed the bio-reactivity of the designed vaccine. The pooled sera from immunized BALB/c mice and the control groups were tested against expressed EM-L2 peptide in *E.coli*. Approximately, 35% higher OD in comparison to control group indicated that an antibody was raised against the EM-L2 recombinant peptide. These results confirmed the phage role as efficient antigen presenting and carrier moiety. It should be noted that we have used a different phagemid as the control group. All of the procedures were performed using the same protocols and materials for both control and test groups. Therefore, it could be deduced that except for the phage vaccines all of the sample contents are common for both groups. Since the samples are only different in their phage content, the reason behind the reduced tumor growth could not be anything other than the employed phage. In order to determine the polarization of immune system towards cellular and humoral immune branches in Prophylactic and Therapeutic groups, the serum levels of specific cytokines were evaluated in both groups. In this regard, the serum levels of IL-4 (as representative cytokine for humoral immunity) and IFN-y (as representative cytokine for cellular immunity) were assessed (data not shown). These evaluations showed that both Humoral and cellular branches of immune system are activated following the immunizations.

To investigate the prophylactic and therapeutic anti-tumor effects of the vaccines, female C57BL/6 inbred mice tumor model was established. Several cell lines have been derived from these tumors like LL/2 cell line,[21] which is known to over express EGFR, and has been used to establish EGFR expressing tumors in several studies.[6,10,32] Analyzing our results, we have derived the conclusion that the phage vaccines have anti-tumor activities. Although our phage vaccination have showed reduced growth rate in the prophylactic mice, the differences were not significant. However, in therapeutic mice significant reduction in tumor growth rate was observed between t-EM-L2 and control groups. These results clearly suggest that therapeutic potency of this novel vaccine is stronger than its prophylactics effects. In our previous study[13] the mimotope-displaying phage vaccine showed no prophylactic properties in mice challenged with LL/2. This phenomenon could be rooted in the size of the displayed mimotope. Since in this study only one copy of the mimotope is presented on the phage particle, there could be some limitation in its surface accessibility due to unfavorable protein folding. Extending the mimotope length by inclusion of its tandem repeats have shown to be effective to enhance the Prophylactic results of the phage vaccine.[14] Ren et al have displayed mFlt4 peptide on T4 phage and reported no significant difference in tumor growth,[33] while the constructed T4 phage displaying mVEGFR showed significant effects on tumor growth rate.[34] Taken together, in light of our results and the previous results, it could be concluded that the size and the type of displayed peptide might be an important factor for better responses. It has been shown that anti-EGFR MAbs ICR61and ICR62 can block the binding of EGF, TGFα, HB-EGF and BTC to the EGFR.[35] Thus, we expect that produced antibodies against EM-L2 would behave like them and compete with EGF to bind the EGFR molecule. Moreover, bacteriophages could be accumulated in tumors and inhibit tumor growth,[32] suggesting that anti-tumor activities of these peptide-phage particles are mediated by the host immune system and mostly throughout antibody production against the displayed peptide. Thus, phage vaccination could be deemed as a potent strategy in cancer therapy.

In conclusion, detached from our results it could be suggested that combining phage and peptide vaccination have significant advantages over conventional strategies. Other than their anti-tumor

properties, immune-modulatory properties of these products might be lucrative in developing peptide-phage-based procedures in cancer studies. Our results revealed that mice immunization by peptide-displaying phage particles may elicit an amenable immune responses and generate effective therapeutic protection against EGFR tumors. EM-L2 peptide is large enough to be considering as an immunogenic peptide provided with an appropriate adjuvant. The potency of this peptide vaccine without the phage particle remained to be elucidated. Although promising results have attained, it should be noted that our study was performed on a limited number of animals which could be extended to higher numbers with more robust and conclusive results.

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