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Abstract

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Immunogenicity and antitumor activity of the superlytic λF7 phage nanoparticles displaying a HER2/neu-derived peptide AE37 in a tumor model of BALB/c mice

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Abstract

Phage display technique has been increasingly researched for vaccine design and delivery strategies in recent years. In this study, the AE37 (Ii-Key/HER-2/neu $_{776–790}$) peptide derived from HER2 (human epidermal growth factor receptor protein) was used as a fused peptide to the lambda phage (λF7) coat protein gpD, and the phage nanoparticles were used to induce antitumor immunogenicity in a TUBO model of breast cancer in mice. Mice were immunized with the AE37 peptide displaying phage, λF7 (gpD::AE37) every 2-week intervals over 6-weeks, then the generated immune responses were evaluated. An induction of CTL immune response by the λF7 (gpD::AE37) construct compared to the control λF7 and buffer groups was observed *in vitro*. Moreover, in the *in vivo* studies, the vaccine candidate showed promising prophylactic and therapeutic effects against the HER2 overexpressing cancer in BALB/c mice.

Keywords: Antitumor Immunogenicity; HER2/neu; Becteriophage λF7; AE37; vaccine; Breast cancer

1. Introduction

Cancer vaccination is a type of immunotherapy in which tumor antigens are presented to the patient's own immune system, via variety of delivery systems, in order to prime/boost an immune response. Several anticancer studies based on phage display technology have been reported [1, 2]. In cancer vaccination, a specific cellular immune response is induced and translated to antitumor activity, delaying tumor growth and resulting in improved survival. Such vaccines may also be used in prophylaxis and therapy of cancers [3, 4]. Peptide-based cancer vaccines are currently under intensive research and research in the field is considered a hot topic exploited by several clinical research protocols. Peptide vaccines designed to combat cancer mechanistically generate a T-cell immune response against tumor in the host [5].

The human epidermal growth factor receptor 2 (HER2) has an intracellular domain with tyrosine kinase activity and is normally expressed during fetal development. The HER2 gene is located on chromosome 17q21 and encodes for a 185-kD transmembrane glycoprotein receptor [6]. Receptor activation by dimerization of its extracellular domain mediates proliferation signaling including PI3K/Akt or MAP kinase pathways, enhancing cell growth, division and survival. This protein is involved both in oncogenesis and tumor survival. Some specific sequences of HER2 are adequately immunogenic which can stimulate cytotoxic T lymphocytes (CTLs). This instructs the cells to recognize and kill cancer cells expressing HER2/neu *in vitro*.

The novel peptide sequence AE37 is a Ii-Key hybrid of the AE36 ($HER2_{776-790}$) derived from the intracellular domain of HER2. It is an MHC class II hybrid molecule, potent to stimulate peptide-specific CD4+ and CD8+ T cells. AE37 is composed of the sequence: Ac-LRMKGVGSPYVSRLLGICL-NH2. This particular peptide sequence of HER2 is 100 %

identical in human, mouse and rat [7-9]. It is safe and is well tolerated as a vaccine. Published reports on its immunological monitoring in human has shown that this immunogenic peptide caused specific long term immunity in most of the evaluated patients [10, 11].

Phage display describes a technique used in biotechnology in which a peptide or protein of interest is genetically fused to a coat protein of a bacteriophage, resulting in the display of the protein fusion on the surface of the phage particle. The DNA encoding the peptide-coat protein fusion may reside in an expression vector expressed in the bacterial host and added to the phage pro-head by genetic complementation [1, 12]. Among its other uses, the phage display technique can also be used to design vaccine adjuvant /delivery systems where an antigenic amino acid sequence can be expressed as a peptide on the phage head [13].

Phages have been used as adjuvant-like particles. Compared to standard vaccination, a vaccination practice using phage particles, requires lower doses of immunogenic molecules but produces higher immunogenicity responses [14, 15]. Hybrid phage vaccines induce effective humoral and cellular responses. In addition to displaying the antigenic molecules, phages may be used as targeting molecules providing significant advantages for application in targeted therapy [16, 17]. Phage vaccines have fundamental properties of the phage nanoparticles including high stability in a broad range of pH and low cost of phage design and production. Moreover, phage nanoparticles cannot proliferate in eukaryotic cells and have minimal side effects in the mammalian host [18].

Bacteriophage lambda (λ) among its other applications, has been used to display immunologic peptides (1). The lytic nature of λ and the conformation of its major capsid protein (gpD) offer several advantages as a phage display candidate. The unique form of the λ capsid and the potential to exploit gpD in design of controlled phage decoration will benefit applications of λ display [19]. The capsid protein gpD, which is necessary for phage viability, has been used extensively for fusion of polypeptides in phage display technique. The DNA sequence representing the capsid-linker-polypeptide can be cloned and expressed from a plasmid in *Escherichia coli*. This protein expressed by the plasmid could be received by λ*Dam15* phage particles infecting the cloned bacteria. λF7 bacteriophage (*λimm21Dam15*) has a mutation (*Dam15*) in the *gpD* gene where glutamine is replaced with a stop codon TAG resulting in a truncated gpD fragment. Thus, the translation of λF7 in a non-suppressor or a wild-type *E. coli* results in unassembled and nonviable phage in the absence of complementing gpD from a plasmid. A functional, wild-type length, gpD protein can be produced with the activity of an amber suppressor strain of *E. coli*, which has the tRNA capability to recognize the stop codon as a specific amino acid [12].

In the current study, an amber suppressor strain of *E. coli* (W3101 SupE) was used for cloning of an expression plasmid containing the gpD capsid-linker-polypeptide (AE37) gene. This strain has the capability to insert glutamine in place of the amber stop codon, producing wild-type gpD. The cloned plasmid used had an ampicillin resistance gene, to avoid proliferation of undesirable strains on medium and for the selction of plasmid containing strains. λF7 could receive the gpD-linker-antigenic polypeptide (AE37) from this plasmid by infecting the amber suppressor strain

(SupE) and therefore surface decorated with AE37 linked to the gpD protein by a linker. We decided to investigate the *in vitro* and *in vivo* immunity induction of the AE37 displaying λ phage nanoparticles in a TUBO breast cancer model of BALB/c mice.

2. Materials and methods

2.1 Bacterial strains, phages and plasmids

Bacterial strains, phages and plasmids used in this study are listed in Table 1. For general purpose phage plating and titration, *Escherichia coli* strain BB4 (*supF58 supE44*) was used. Plasmid pGPD::AE37 was constructed using plasmid pPL451-gpD. The procedure was based on the protocols reported in previous studies [12, 20]. To produce the fusion peptide, the terminal stop codon from gpD was removed and an in-frame fusion with the AE37 sequence was created. The two fragments were separated by an in-frame short linker encoding 17 amino acids (ACTAGCGGGTTCTGGTTCCGGTTCTGGTTCCGGTTCTGGC) that was placed between and followed by a *Kpn*I cut site to maximize fusion functionality and also allow for additional fusions to be designed in the future. The gpD-linker-AE37 sequence was then amplified and cloned into the *Hpa*I and *Nco*I sites on pGPD, placing it under the control of the P_L strong promoter that is regulated by the temperature-inducible λ repressor *C*I857 that confers temperature-regulated expression [21].

2.2 Phage lysate preparation

Cultures of transformed *E. coli* strain (*SupE*) (pGPD::AE37) were grown on LB agar plates with ampicillin and incubated overnight at 37 °C. Dilutions of primary lysates (1:1000) were prepared in 100 µL of TN buffer (0.01 M Tris–HCl and 0.1 M NaCl, pH 7.8), (Fisher Scientific, USA). Lysate dilutions were added to 500 µL of cells ($1x10^8$ CFU/mL), incubated for 2 hours at experimental room temperature prior to adding 5 ml of top LB agar (LB broth + 0.7 % agar, Bacto Agar from Difco Laboratories, Sparks, MD). The plates were then incubated overnight at 37 °C. Plate lysates were prepared by adding 10 mL of ice cold sterile TN buffer to the surface of the plate, incubating overnight at 4 °C. The top agar was scratched by a sterile loop and the resulting solution with the loosened top agar were then transferred aseptically to a conical tube, mixing and centrifuging at 8000 RPM (Hettich, Germany) for 20 min at 4 °C. The resulting supernatant was then poured in a fresh ice-cold conical tube and 2 µL of chloroform was added to kill the remaining host bacteria. Lysates were then precipitated by centrifuging at 8000 RPM for 10 min at 4 °C. The supernatant was removed and transferred into a new sterile tube. Then 1 µL DNase (Sina Colon, IRAN) was added to the lysate to remove any remaining free DNA in the lysate. The lysates were then passed through a 0.45 µm filter and kept at 4 ^{o}C until use.

2.4 Phage purification

Polyethylene glycol (PEG)-8000 (Fisher Scientific, USA) was added to a final concentration of 10 % (w/v). The bacteriophage particles were then recovered by centrifugation at 8000 RPM for 10 min at 4 °C. The supernatant was discarded and 1 ml TN buffer was added to the pellet and kept overnight at 4 °C. To separate PEG and cell debris from the phage nanoparticles, an equal volume of chloroform was added. The mixture was mixed gently for 30 seconds and spun at 4300 RPM for 15 min at 4 °C. The aqueous phase, which contained the bacteriophage particles, was then removed. The solution was filtered through a sterile 0.45 µm syringe filter (BD Discardit, India). To remove endotoxin (LPS), 1% Triton X-114 was added and the solution was incubated in a shaker incubator for 30 min at 4 °C (Innova 4080 Incubator Shaker). Then the solution was incubated at 37 °C for 10 min before centrifugation at 14000 RPM for 10 min at 25 °C. In order to maximize endotoxin elimination, the phage purification procedure was repeated three times. The phage solutions were tittered at each step of purification by standard viability assays on fresh Sup^+ BB4 (*supE, supF*) *E. coli* cells as these cells have been reported to consistently produce the highest titers of λF7 [19]. For endotoxin content evaluation, the samples were analyzed by a standard Limulus Amebocyte Lysate (LAL) assay (Samen Research Institute, Samen Pharmaceutical Co. Mashhad, Iran). Samples were kept at 4 °C until use.

2.5 Animals and cell lines

Female BALB/c mice (four to six weeks old) were purchased from Pasteur Institute (Tehran, Iran). All the protocols were approved by the Ethical and Research Advisory Committee, Mashhad University of Medical Sciences (MUMS), according to animal welfare guidelines (Project code: MUMS 922610).

TUBO cell line which overexpresses the rat HER2/neu protein (rHER2) was kindly provided by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy) and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 20 % fetal bovine serum (FBS). A murine colon carcinoma cell line, CT26, which does not express HER2 was purchased from Pasteur Institute (Tehran, Iran) and cultured in RPMI-1640 medium supplemented with 10 % FBS and served as a negative control.

2.6 Immunization of BALB/c mice

The immunization procedure was performed for three times at two week intervals. BALB/c mice were divided into three experimental groups (10 mice in each group). The mice in the test group were injected with 100 µL of 10^8 (PFU/mL) AE37 displaying phage nanoparticles [λF7

(gpD::AE37)] subcutaneously (SC). For control groups, either 100 µL of 10^8 (PFU/mL) λF7 or 100 µL TN buffer was injected (SC).

2.7 Analysis of T-Cell immune responses (the extracellular cytokine assay)

ELISA assay was performed using anti mouse IF-γ and anti-mouse IL-4 ELISA kits. According to the manufacturer's instruction one day before the test, two ELISA 96-well plates were coated with mouse anti-IL-4 and anti-IFN-γ antibodies. Plates were incubated at 4º C overnight. Serum and splenocytes of the mice (three mice from each group) were collected for evaluation the amounts of IL-4 and IFN-γ according to the instruction (eBioscience, San Diego, CA, USA). All assays were performed in triplicate. Phytohaemagglutinin (PHA) was used for stimulation production of cytokine as positive control.

2.8 In vitro CTL assay

Two weeks after the final vaccination, mice were sacrificed and splenocytes harvested by ammonium chloride lysis buffer (NH_4Cl, 0.1 M and Tris, 0.2 M). Viable splenocytes were counted using trypan blue (0.4 %, w/v) and re-stimulation was performed with the 10^8 PFU AE37 displaying phages (100 µL). TUBO cells (target cells) were incubated with 12.5 µM Calceine AM (Calcein-AM, Invitrogen, USA) at 37 °C for one hour in the dark [22]. Triton X-100 (2 %) and culture medium were added to the maximum and minimum release wells respectively. Fluorescence intensity was measured at 485 nm (excitation) and of 538 nm (emission) using a fluorescent plate reader (FLX 800, BioTek Instruments Inc. USA). The percentage of specific lysis was calculated by the following formula: (release by CTLs - minimum release by targets)/ (maximum release by targets - minimum release by targets) × 100 [23, 24]. To show the specificity of cytotoxic activity, non-expressing rHER2/neu, CT26 cells were used as negative controls.

2.9 Prophylactic Model of TUBO Challenge

Fourteen days after the last vaccination, 5×10^5 TUBO cells in 50 µL PBS buffer were injected SC in the right flank of immunized mice (seven mice per group). Mice were monitored every day. Three orthogonal diameters of the developing tumor (a, b, c) were measured with a digital caliper. The tumor volumes were calculated according to the formulation [(height × width × length) × 0.5]. The equation of the line obtained by exponential regression of the tumor growth curve was used for TTE (time to reach the end point) and based on the difference between the median TTE of treatment group (T) and the median TTE of the control group (C) were used to calculate the percent TGD (the percent of tumor growth delay) (TGD % = [(T- C) /C] × 100]) for each mouse. For ethical reasons, mice were sacrificed if the following conditions observed: the

tumor volume was greater than 1000 mm^3, the body weight loss was over 15 % of initial weight or the mice became sick and unable to feed.

2.10 Therapeutic Model of TUBO Challenge

To evaluate the anti-tumor efficacy of AE37 displaying phages and control λF7, 5×10^5 TUBO cells in 50 µL PBS buffer were injected in the right flank of 4-6 week old female BALB/c mice. Two weeks after tumor inoculation, 10^8 PFU of AE37 displaying phages, λ F7 or TN buffer (100 µL/mouse) were injected subcutaneously (SC) three times at 2-week intervals. The TN buffer and λF7 were used as controls. Mice without any tumor considered as tumor-free at the end of the experiment. Again, mice were euthanized if the tumor volume was greater than 1000 mm^3, or the body weight reached below 15% of initial mass or the mice became lethargic or sick or unable to feed. Mice were monitored every day and the tumor volume was measured and calculated as mentioned above.

2.11 Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey's post-test were performed to assess the significance of the differences among various formulations. Survival data expressed as survival probability was analyzed by log-rank test to compare survival curve between groups. Results with $P < 0.05$ were considered significant. All statistical analyses were performed using Graph Pad Prism 6 Software. * Means *P*. value < 0.05, ** means *P*. value < 0.01, *** means *P*. value < 0.001, **** means *P*. value < 0.0001.

3. Results

3.1 Endotoxin removal from phage lysates

Triton X-114 was used for endotoxin removal. In order to maximize endotoxin elimination, the phage purification procedure was repeated three times. LAL results showed that samples contained < 12 EU/mL. This amount is considered an injectable grade of endotoxin level for animals [25].

3.2 Determination of CD8+ T-cells by flow cytometry

The percentage of $CD8^+$ T cells within the $CD8^+$lymphocyte population was significantly enhanced in mice injected with λF7 (gpD::AE37) compared to controls groups ($P < 0.05$) (Fig. 1).

3.3 Antigen-specific cytotoxicity by λF7 (gpD::AE37)

Cytotoxicity assays provide an *in vitro* evaluation of the lytic activity of T cells against tumors [26]. The λF7 (gpD::AE37) phage was significantly effective in generating CTL response. The activity was established significantly at both various effector to target (E/T) ratio (at 2.5/1 and 10/1) in comparison with the λF7 and TN buffer groups ($P < 0.0001$) and ($P < 0.001$). This response was antigen specific because the CTL response was not observed against CT26 tumor cells (rHER2/neu negative) (Fig. 2).

3.4. IL-4 and IFN-γ production assays

The sera of immunized mice (three animals per group) were collected 14 days after the last booster and assayed for IL-4 and IFN-γ by ELISA. Mice stimulated with λF7 (gpD::AE37) secreted higher levels of IL-4 ($P < 0.01$) and IFN-γ compared to λF7 and buffer groups (Fig. 3).

3.5 Prophylactic study

10^8 PFU of AE37 displaying phages and λ F7 (100 µL/mouse) were used to vaccinate mice subcutaneously at two week intervals. Two weeks after the third vaccination all groups were challenged with 5×10^5 TUBO cells by injection on right flank and the mice were observed for any touchable tumor on the right flank. Tumor growth curve analysis indicated that the λF7 (gpD::AE37) and λF7 groups were the most effective groups in terms of reducing the growth rate of the tumor ($P < 0.0001$) and ($P < 0.001$) in comparison to TN buffer (Fig. 4.A). The prophylactic effects observed in mice model groups are summarized in Table 2 indicating median survival time (MST), time to reach end point (TTE) and tumor growth delay (% TGD) for each mice group.
Survival analysis revealed that the λF7 (gpD::AE37) and λF7 group had significantly prolonged MST, TTE and % TGD compared to the TN buffer ($P < 0.01$) (Fig. 4.B).

3.6 Therapeutic study

In the therapeutic evaluation study, 5×10^5 TUBO cells per mice were subcutaneously injected in the right flank of experimental and control animals. After observation of a palpable tumor, vaccination was started for three times with two week intervals. Weights of mice and sizes of tumors were measured regularly until mice became lethargic or size of tumors was reached up to 1000 mm^3. The candidate vaccine formula λF7 (gpD::AE37) decreased the size of tumor

significantly ($P < 0.001$) and λF7 group ($P < 0.05$) in comparison with TN buffer (Fig. 5. A). In the λF7 (gpD::AE37) vaccination group an increased survival time was observed ($P < 0.05$) which in comparison with λF7and TN buffer groups was statistically significant (Fig. 5. B). The therapeutic effects observed in mice model groups are summarized in Table3 indicating median survival time (MST), time to reach end point (TTE) and tumor growth delay (% TGD) for each mice group.

4. Discussion

The goal of our study was to investigate the immunogenicity and anti-tumor activity of the chimeric λ phage nanoparticles displaying immunogenic AE37 peptide, λF7 (gpD::AE37) in a TUBO tumor model of BALB/c mice. Cancer peptide vaccines, based on tumor-associated antigens (TAA), can induce cellular and humoral immune response against the tumor or they may cause an enhancement of an endogenous antitumor immunity pre-existing in the host [27]. HER2/neu is a receptor belonging to the epidermal growth factor receptor family the overexpression of which has been observed in 18–20% of human breast cancers and linked to a poor prognosis [28].

In the last few years, different HER2/neu-derived epitopes have been targeted simultaneously, leading to a heightened response. AE37 is a hybrid peptide which is composed of the covalent linkage of the Ii-Key peptide (LRMK), to the HER2 derived AE36 amino-terminus [29]. Some studies have shown that Ii-Key hybrid peptide, enhanced the presentation of antigenic peptides by APCs to T cells, stimulated peptide-specific $CD4^+$ T cells more impressive than native peptides and provided potent helper effect to HER2-specific $CD8^+$ T cells in animal models [30]. The AE37 peptide vaccine, with the LRMK sequence, can facilitate MHC class II molecule loading and increased potency compared with unmodified class II epitopes [31]. It has been reported that AE37 is a multi epitope vaccine. It is capable of inducing both specific CD4+ and CD8+ T cells in vaccinated cancer patients [32]. It has been hypothesized that AE37-induced T-helper cells may engage dendritic cells at tumor site, thereby cross-presenting antigens from apoptotic tumor cells and inducing epitope spreading [33] . Immunization with a HER-2 helper peptide could elicit tumor specific CTLs via cross-presentation [7]. The induced immune responses might be directed against the targeted epitope as well as against a broad range of tumor associated epitopes [9, 10]. Moreover, the AE37 peptide stimulates CD4+ Th cells rendering them capable of inducing immunologic memory and persistent stimulation of CTLs. AE37 induced T cells, secreting mainly Th1 cytokines, may activate dendritic cells present in tumor microenvironment. Under these conditions the cross-presentation phenomenon could be enhanced resulting in an epitope spreading [11].

Furthermore, phase II trial investigating AE37 + GM-CSF based vaccines have shown its effectiveness in stimulating peptide-specific immunity, especially in CD8+ T cell stimulation with anti-tumor activity in breast cancer patients but these patients experienced grade 1 local and systemic toxicity due to the GM-CSF. The most common systemic toxicities have been influenza-like symptoms, fatigue and bone pain [34].

A nanoliposomal AE36 vaccine with CpG adjuvant has shown a considerable effect in prophylactic and therapeutic studies in mice [35]. Designing suitable delivery systems based on immune-stimulating complexes that have a long circulation time and their tendency to be taken up more efficiently by APCs to induce CTL response can therefore provide considerable improvement in vaccination [36]. Due to the several great beneficial features including large multivalent display, ease of manufacture, excellent safety profile and intrinsic adjuvant activity the phage display technique is on the focus for a rapid development for anticancer vaccine development and cancer vaccine delivery [37].

The lambda phage vector is about 1000 times more efficient than a plasmid vector to transform a target peptide molecule. It can be converted to a plasmid for the production of foreign peptides and proteins. Moreover, the ease of plaque screening and the efficiency of infection with lambda is evident [38]. In a recent study, we have shown that λF7 (gpD::E75) particles displaying the E75 peptide (another HER2 derived molecule), can stimulate specific CD8+ T cells *in vitro* [1]. Lambda and T7 phage display systems have shown to be able to elicit a B cell response in cancer cells [39]. Recombinant λ phage nanoparticles carrying HBsAg (hepatitis B surface antigen) stimulated specific antibodies production in rabbits and mice [40]. The peptide inoculant λ gfp10-GFP-TAT and hybrid DNA could induce the greatest amplitude of an IFN-γ production in CD1 mice [41]. Phages displaying the melanoma antigen ($MAGE_{161–169}$) produced significant CTL immune responses against a tumor-associated antigen epitope leading to therapeutic and protective effects in a C57BL/6J mice model [42].

In this study, we employed λF7 phage particles displaying the AE37 peptide (AE36, $HER2_{776-790}$ linked to LRMK peptide) on λF7 (gpD::AE37). The immunogenicity and antitumor potential of the λF7 (gpD::AE37) was investigated using *in vivo* and *in vitro* assays. BALB/c mice were immunized subcutaneously three times with endotoxin-free λF7 (gpD::AE37) phage nanoparticles. Our data demonstrated that the *λ* phage could act as an endogenous adjuvant. The bacterial pathogen-associated molecular patterns (PAMPs) probably act as an endogenous adjuvant. The observed immunogenicity of the control phage in the prophylactic and therapeutic settings has been reported consistently in our other projects including one of our recently published studies [43]. The effects in the prophylactic setting may even be higher due to longer exposure time. Higher prophylactic (compared to therapeutic) effects of bacteriophages has been reported in other settings [44]. It has also been reported by many investigators that phage particles are inherently immunogenic and can serve as effective natural adjuvants. This is why phage display vaccines may be effective without adjuvants that are frequently used along with recombinant proteins and synthetic peptides to improve immune response [45]. This inherent immunogenicity is considered a great advantage for the phage vaccines in addition to their many

other advantages including their high multivalent display potential, safety profile, and ease of manufacturing and construction [46, 47]. Therefore, the observed immunogenicity by the empty phage particles are expected to be seen. The flow cytometry assays showed that mice immunized with λF7 (gpD::AE37) nanoparticles could induce a significant $CD8^+$ T-cell response compared to control groups. In addition, cytotoxicity assays showed that mice immunized with λF7 (gpD::AE37) nanoparticles could induce a higher CTL activity compared to λF7 and buffer groups indicating the crucial role of the repetitive display of AE37 peptide on the surface of λ nanoparticles. *In vivo* studies demonstrated that λF7 (gpD::AE37) nanoparticles decreased tumor growth and possessed superior anti-tumor activity in both prophylactic and therapeutic assays. Also, it was able to increase the survival time of the TUBO cell line (HER2-over expressing) tumor bearing mice. We also report that λF7 successfully induced tumor-specific CTL activity immune responses in comparison to the TN buffer. The control λF7 did not show i*n vitro* induction of CD8+ T-cell and CTL activity.

In summary, the results of this study demonstrates that vaccination with lambda phage nanoparticles expressing AE37 peptide, λF7 (gpD::AE37) led to the induction of specific CTL immune response *in vitro* and *in vivo*. Moreover, the delivery of AE37 peptide by λ bacteriophage has significantly enhanced the anti-tumor immune function compared to the control groups. In fact, we think that there is a potential for the λF7 (gpD::AE37) phage nanoparticles for antitumor vaccine development as the phage construct was found to elicit inhibitory effects on the TUBO tumor (HER2/neu overexpressing implantable tumor) and therefore we think that our data merits further investigation. Further studies into the exploitation of the vaccine candidate is underway.

5. Conflicts of interest

The authors declare no conflicts of interest.

6. Acknowledgements

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Table 1**.** Bacterial strains, plasmids and phages used in this study

Designation	Genotype	Source/Reference
Bacterial strains		
BB4	*supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA* DE(*lac*) U169	Agilent Technologies, Inc
W3101	F−, *galT22*, λ−, *IN(rrnD-rrnE)1*, *rph-1*	CGSC #4467, Bachmann *et al* (Bachmann, 1972)
W3101 SupE	F−, *galT22*, λ−, *IN(rrnD-rrnE)1*, *rph-1 crcA280::Tn10*, *glnV44*(AS)	Nicastro *et al* (Nicastro, et al., 2013)
Phages		
λF7	*λDam15imm21CIts*	Mikawa *et al* (Mikawa, et al., 1996)
λF7 (gpD::AE37)	*λDam15imm21CIts*	This study
Plasmids		
pPL451gpD	*pM-cI857-pL-cI857-pL- D-tL*	Sokolenko *et al* (Sokolenko, et al., 2012)
pGPD::AE37	*pM-cI857-pL-cI857-pL- D::E37-tL*	This study

Table 2: Protective efficacy data in TUBO tumor mice model (n = 7)

Formulation	MST[a] (Day)	TTE[b] (Day) ± SD	TGD[c] (%)
λF7 (gpD::AE37)	98	99 ± 20	62
λF7	90	92 ± 12	51
TN buffer	67	61 ± 33	-

[a] Median survival time

[b] Time to reach end point

[c] Tumor growth delay

Table 3: Therapeutic efficacy data of the vaccine in TUBO tumor mice model (n = 7).

Formulation	MST[a] (Day)	TTE[b] (Day) ± SD	TGD[c] (%)
λF7(gpD::AE37)	60	64 ± 1	115
λF7	43	52 ± 9	70
TN buffer	44	30 ± 6	-

[a] Median survival time

[b] Time to reach end point

[c] Tumor growth delay

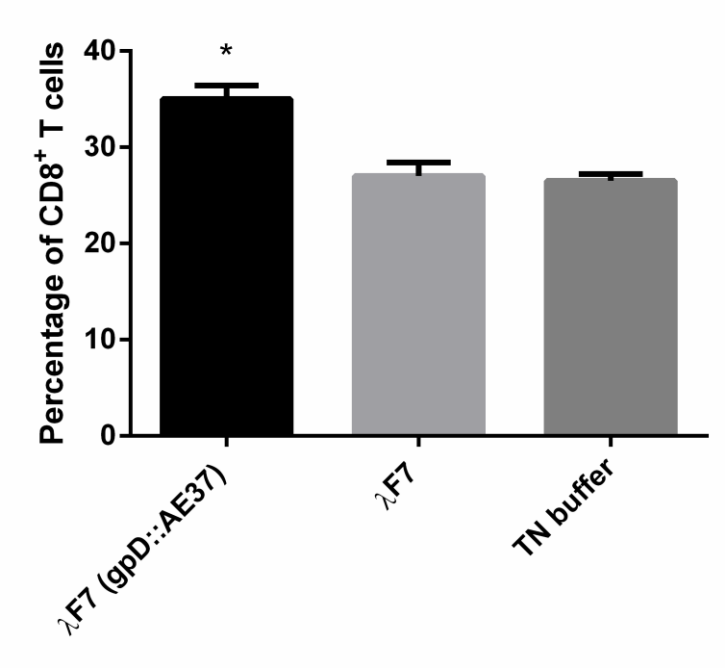

Fig. 1. Flow cytometry assay with splenocytes of immunized mice. Fourteen days after the last immunization, splenocytes were isolated and stimulated *in vitro* with PMA/I for 4 h and stained with a surface CD8. Percentage of CD8+ T cells significantly increased in λF7 (gpD::E37) group compared to λF7 and TN buffer groups *($P < 0.05$). The results represent mean ± SEM (n = 3).

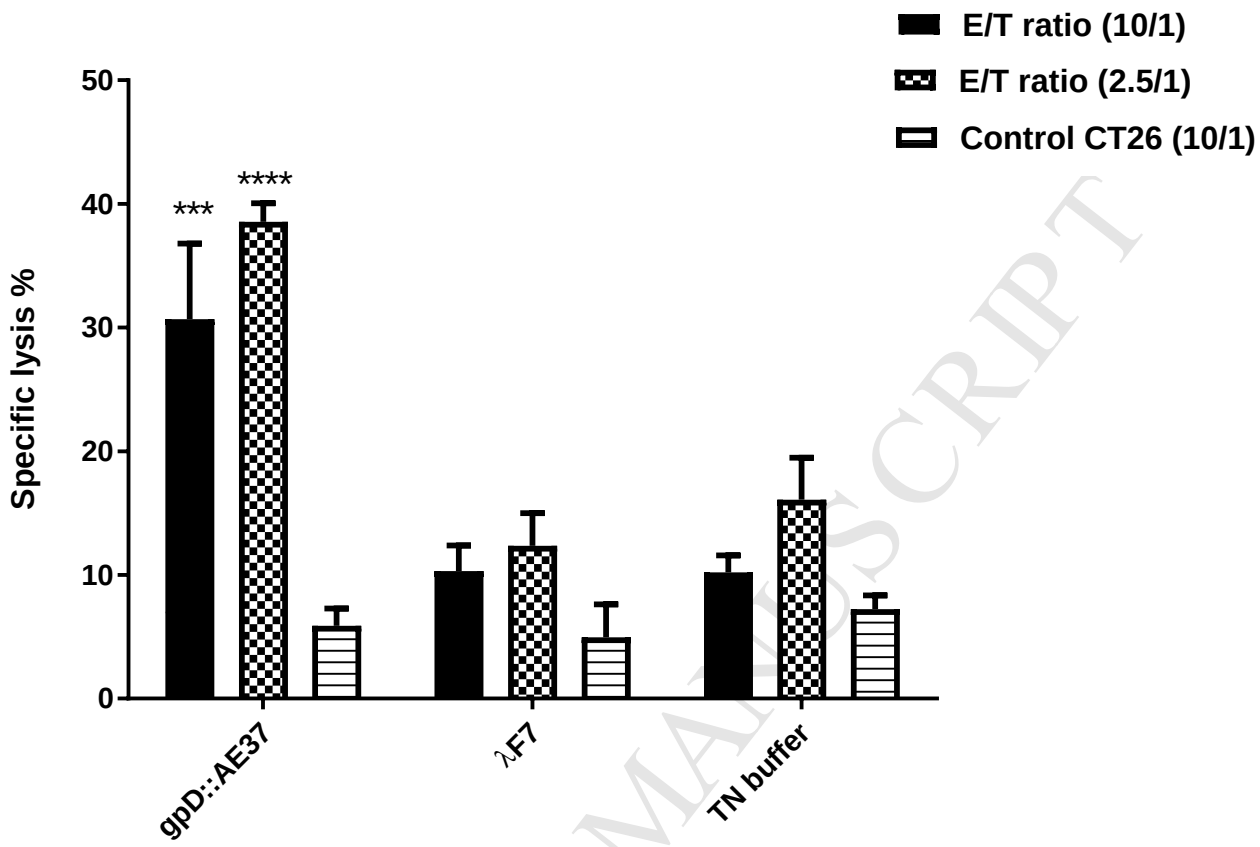

Fig. 2. Antigen - Specific CTL response induced by various formulations at two different ratios of effector to target cells (E/T) was assessed using an *in vitro* CTL activity assay. Splenocytes from the mice (from three mice in each group) were incubated with Calcein AM-loaded rHER2/neu-expressing TUBO tumor cells and rHER2/neu-expressing negative CT26 cells (as rHER2/neu negative control). The mice immunized with λF7 (gpD::E37) showed significantly higher CTL activity compared to λF7 and buffer groups at E/T ratios. ****P* <0. 001 and *****P* <0.0001. The results represent mean ± SEM (n = 3).

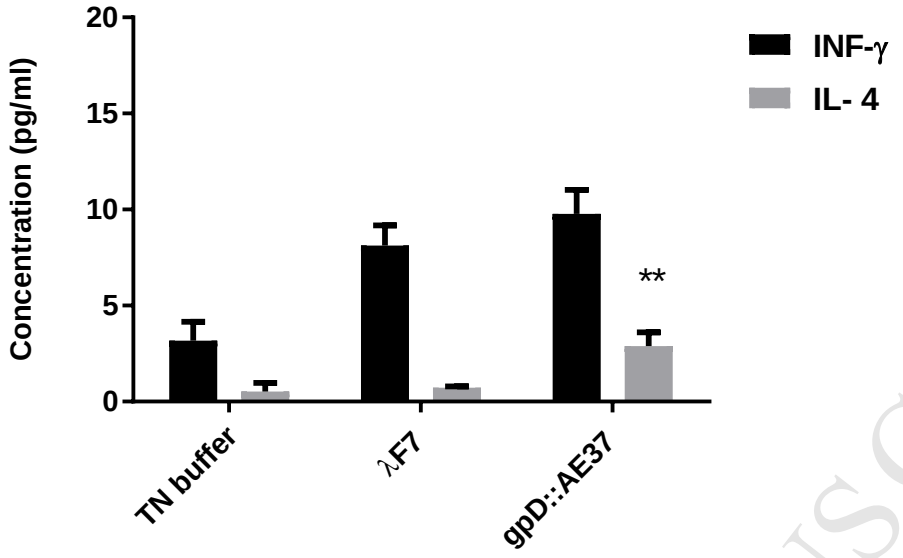

Fig. 3. Secretion of IL-4 and IFN-γ cytokines induced by phages expressing AE37 peptide. BALB/c mice were immunized with λF7 (gpD::AE37) every 2 weeks for three times. Blood samples were collected 14 days after the last booster and the concentration of IL-4 and IFN-γ cytokines was determined using ELISA. Mice immunized with λF7 (gpD::AE37) showed higher levels of IL-4 and IFN-γ cytokines compared to λF7 and buffer groups. Data represent mean ± SD (n = 3). **(P < 0. 01)

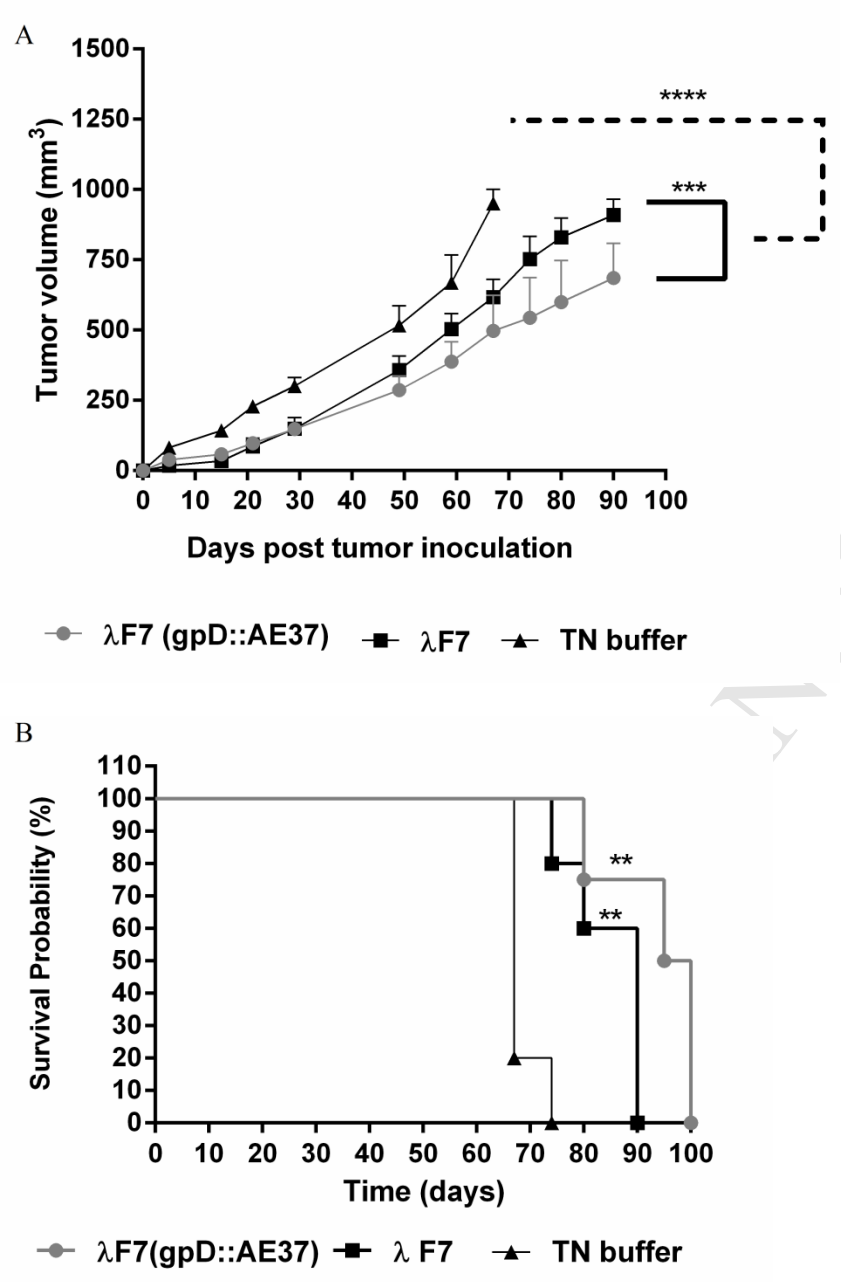

Fig. 4. Protective effects of vaccination with λF7 (gpD::AE37) phage in BALB/c mice against a TUBO tumor model. Two weeks after the last booster, seven mice in each group were challenged subcutaneously on right flank with 5 ×10^5 TUBO cells. Mice were observed for tumor growth (A) and survival (B). Tumor size was calculated twice per week, based on the three dimensions. The survival of mice was followed for 100 days. The data indicate mean ± SEM (n = 7). ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$; denotes significant difference from the TN buffer and control groups.

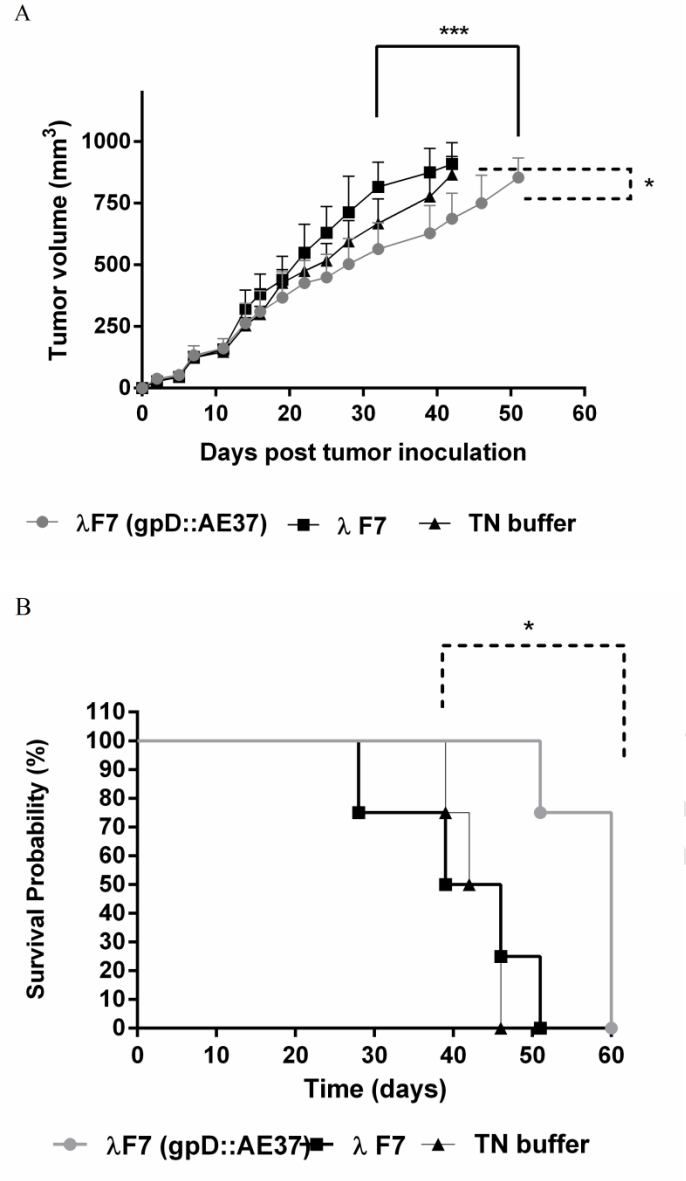

Fig. 5. Therapeutic effects of λF7 (gpD::AE37) phage nanoparticles against the HER2 expressing TUBO tumor of BALB/c mice. Two weeks after injection of 5 $\times 10^5$ TUBO cells (seven mice in each group) the mice were administrated with the samples for three times at two week intervals. After the first injection, the mice were challenged and tumor size was calculated based on the three dimensions. (A) Tumor growth was measured twice per week. (B) Mice survival was followed for 74 days. The data indicate mean ± SEM (n = 7). *$P < 0.05$ denotes significant effects compared to the TN buffer group.

- Bacteriophage λ displaying the HER2/neu derived peptide AE37

- Immunological studies on TUBO cancer cell line and mice bearing HER2+ breast cancer.

- Prophylactic and therapeutic studies on mice bearing HER2+ breast cancer.