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Immunogenicity and antitumor activity of the superlytic λ F7 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 (li-Key/HER-2/neu₇₇₆₋₇₉₀) peptide derived from HER2 (human epidermal growth factor receptor protein) was used as a fused peptide to the lambda phage (λ F7) 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, λ F7 (gpD::AE37) every 2-week intervals over 6-weeks, then the generated immune responses were evaluated. An induction of CTL immune response by the λ F7 (gpD::AE37) construct compared to the control λ F7 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.

1 **Immunogenicity and antitumor activity of the superlytic λ F7 phage nanoparticles**
2 **displaying a HER2/neu-derived peptide AE37 in a tumor model of BALB/c mice**

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46 Abstract

47
48 Phage display technique has been increasingly researched for vaccine design and delivery
49 strategies in recent years. In this study, the AE37 (Ii-Key/HER-2/neu₇₇₆₋₇₉₀) peptide derived
50 from HER2 (human epidermal growth factor receptor protein) was used as a fused peptide to the
51 lambda phage (λ F7) coat protein gpD, and the phage nanoparticles were used to induce antitumor
52 immunogenicity in a TUBO model of breast cancer in mice. Mice were immunized with the
53 AE37 peptide displaying phage, λ F7 (gpD::AE37) every 2-week intervals over 6-weeks, then the
54 generated immune responses were evaluated. An induction of CTL immune response by the λ F7
55 (gpD::AE37) construct compared to the control λ F7 and buffer groups was observed *in vitro*.
56 Moreover, in the *in vivo* studies, the vaccine candidate showed promising prophylactic and
57 therapeutic effects against the HER2 overexpressing cancer in BALB/c mice.

58
59 **Keywords:** Antitumor Immunogenicity; HER2/neu; Bacteriophage λ F7; AE37; vaccine; Breast
60 cancer

63 1. Introduction

64
65 Cancer vaccination is a type of immunotherapy in which tumor antigens are presented to the
66 patient's own immune system, via variety of delivery systems, in order to prime/boost an
67 immune response. Several anticancer studies based on phage display technology have been
68 reported [1, 2]. In cancer vaccination, a specific cellular immune response is induced and
69 translated to antitumor activity, delaying tumor growth and resulting in improved survival. Such
70 vaccines may also be used in prophylaxis and therapy of cancers [3, 4]. Peptide-based cancer
71 vaccines are currently under intensive research and research in the field is considered a hot topic
72 exploited by several clinical research protocols. Peptide vaccines designed to combat cancer
73 mechanistically generate a T-cell immune response against tumor in the host [5].

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

82 The novel peptide sequence AE37 is a Ii-Key hybrid of the AE36 (HER2₇₇₆₋₇₉₀) derived from the
83 intracellular domain of HER2. It is an MHC class II hybrid molecule, potent to stimulate
84 peptide-specific CD4⁺ and CD8⁺ T cells. AE37 is composed of the sequence: Ac-
85 LRMKGVGSPYVSRLGICL-NH₂. This particular peptide sequence of HER2 is 100 %

86 identical in human, mouse and rat [7-9]. It is safe and is well tolerated as a vaccine. Published
87 reports on its immunological monitoring in human has shown that this immunogenic peptide
88 caused specific long term immunity in most of the evaluated patients [10, 11].
89 Phage display describes a technique used in biotechnology in which a peptide or protein of
90 interest is genetically fused to a coat protein of a bacteriophage, resulting in the display of the
91 protein fusion on the surface of the phage particle. The DNA encoding the peptide-coat protein
92 fusion may reside in an expression vector expressed in the bacterial host and added to the phage
93 pro-head by genetic complementation [1, 12]. Among its other uses, the phage display technique
94 can also be used to design vaccine adjuvant /delivery systems where an antigenic amino acid
95 sequence can be expressed as a peptide on the phage head [13].
96 Phages have been used as adjuvant-like particles. Compared to standard vaccination, a
97 vaccination practice using phage particles, requires lower doses of immunogenic molecules but
98 produces higher immunogenicity responses [14, 15]. Hybrid phage vaccines induce effective
99 humoral and cellular responses. In addition to displaying the antigenic molecules, phages may be
100 used as targeting molecules providing significant advantages for application in targeted therapy
101 [16, 17]. Phage vaccines have fundamental properties of the phage nanoparticles including high
102 stability in a broad range of pH and low cost of phage design and production. Moreover, phage
103 nanoparticles cannot proliferate in eukaryotic cells and have minimal side effects in the
104 mammalian host [18].
105 Bacteriophage lambda (λ) among its other applications, has been used to display immunologic
106 peptides (1). The lytic nature of λ and the conformation of its major capsid protein (gpD) offer
107 several advantages as a phage display candidate. The unique form of the λ capsid and the
108 potential to exploit gpD in design of controlled phage decoration will benefit applications of λ
109 display [19]. The capsid protein gpD, which is necessary for phage viability, has been used
110 extensively for fusion of polypeptides in phage display technique. The DNA sequence
111 representing the capsid-linker-polypeptide can be cloned and expressed from a plasmid in
112 *Escherichia coli*. This protein expressed by the plasmid could be received by λ Dam15 phage
113 particles infecting the cloned bacteria. λ F7 bacteriophage (λ imm21Dam15) has a mutation
114 (Dam15) in the gpD gene where glutamine is replaced with a stop codon TAG resulting in a
115 truncated gpD fragment. Thus, the translation of λ F7 in a non-suppressor or a wild-type *E. coli*
116 results in unassembled and nonviable phage in the absence of complementing gpD from a
117 plasmid. A functional, wild-type length, gpD protein can be produced with the activity of an
118 amber suppressor strain of *E. coli*, which has the tRNA capability to recognize the stop codon as
119 a specific amino acid [12].
120 In the current study, an amber suppressor strain of *E. coli* (W3101 SupE) was used for cloning of
121 an expression plasmid containing the gpD capsid-linker-polypeptide (AE37) gene. This strain
122 has the capability to insert glutamine in place of the amber stop codon, producing wild-type gpD.
123 The cloned plasmid used had an ampicillin resistance gene, to avoid proliferation of undesirable
124 strains on medium and for the selection of plasmid containing strains. λ F7 could receive the gpD-
125 linker-antigenic polypeptide (AE37) from this plasmid by infecting the amber suppressor strain

166

167

168 **2.4 Phage purification**

169

170 Polyethylene glycol (PEG)-8000 (Fisher Scientific, USA) was added to a final concentration of
171 10 % (w/v). The bacteriophage particles were then recovered by centrifugation at 8000 RPM for
172 10 min at 4 °C. The supernatant was discarded and 1 ml TN buffer was added to the pellet and
173 kept overnight at 4 °C. To separate PEG and cell debris from the phage nanoparticles, an equal
174 volume of chloroform was added. The mixture was mixed gently for 30 seconds and spun at
175 4300 RPM for 15 min at 4 °C. The aqueous phase, which contained the bacteriophage particles,
176 was then removed. The solution was filtered through a sterile 0.45 µm syringe filter (BD
177 Discardit, India). To remove endotoxin (LPS), 1% Triton X-114 was added and the solution was
178 incubated in a shaker incubator for 30 min at 4 °C (Innova 4080 Incubator Shaker). Then the
179 solution was incubated at 37 °C for 10 min before centrifugation at 14000 RPM for 10 min at 25
180 °C. In order to maximize endotoxin elimination, the phage purification procedure was repeated
181 three times. The phage solutions were tittered at each step of purification by standard viability
182 assays on fresh Sup⁺ BB4 (*supE*, *supF*) *E. coli* cells as these cells have been reported to
183 consistently produce the highest titers of λF7 [19]. For endotoxin content evaluation, the samples
184 were analyzed by a standard Limulus Amebocyte Lysate (LAL) assay (Samen Research Institute,
185 Samen Pharmaceutical Co. Mashhad, Iran). Samples were kept at 4 °C until use.

186

187

188 **2.5 Animals and cell lines**

189

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

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

200

201 **2.6 Immunization of BALB/c mice**

202

203 The immunization procedure was performed for three times at two week intervals. BALB/c mice
204 were divided into three experimental groups (10 mice in each group). The mice in the test group
205 were injected with 100 µL of 10⁸ (PFU/mL) AE37 displaying phage nanoparticles [λF7

206 (gpD::AE37)] subcutaneously (SC). For control groups, either 100 μ L of 10^8 (PFU/mL) λ F7 or
207 100 μ L TN buffer was injected (SC).

208

209 **2.7 Analysis of T-Cell immune responses (the extracellular cytokine assay)**

210

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

218

219 **2.8 In vitro CTL assay**

220

221 Two weeks after the final vaccination, mice were sacrificed and splenocytes harvested by
222 ammonium chloride lysis buffer (NH_4Cl , 0.1 M and Tris, 0.2 M). Viable splenocytes were
223 counted using trypan blue (0.4 %, w/v) and re-stimulation was performed with the 10^8 PFU
224 AE37 displaying phages (100 μ L). TUBO cells (target cells) were incubated with 12.5 μ M
225 Calcein AM (Calcein-AM, Invitrogen, USA) at 37 °C for one hour in the dark [22]. Triton X-
226 100 (2 %) and culture medium were added to the maximum and minimum release wells
227 respectively. Fluorescence intensity was measured at 485 nm (excitation) and of 538 nm
228 (emission) using a fluorescent plate reader (FLX 800, BioTek Instruments Inc. USA). The
229 percentage of specific lysis was calculated by the following formula: (release by CTLs -
230 minimum release by targets) / (maximum release by targets - minimum release by targets) \times 100
231 [23, 24]. To show the specificity of cytotoxic activity, non-expressing rHER2/neu, CT26 cells
232 were used as negative controls.

233

234 **2.9 Prophylactic Model of TUBO Challenge**

235

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

245 tumor volume was greater than 1000 mm³, the body weight loss was over 15 % of initial weight
246 or the mice became sick and unable to feed.

247

248 **2.10 Therapeutic Model of TUBO Challenge**

249

250 To evaluate the anti-tumor efficacy of AE37 displaying phages and control λ F7, 5×10^5 TUBO
251 cells in 50 μ L PBS buffer were injected in the right flank of 4-6 week old female BALB/c mice.
252 Two weeks after tumor inoculation, 10^8 PFU of AE37 displaying phages, λ F7 or TN buffer (100
253 μ L/mouse) were injected subcutaneously (SC) three times at 2-week intervals. The TN buffer
254 and λ F7 were used as controls. Mice without any tumor considered as tumor-free at the end of
255 the experiment. Again, mice were euthanized if the tumor volume was greater than 1000 mm³, or
256 the body weight reached below 15% of initial mass or the mice became lethargic or sick or
257 unable to feed. Mice were monitored every day and the tumor volume was measured and
258 calculated as mentioned above.

259

260

261 **2.11 Statistical analysis**

262

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

269

270

271

272 **3. Results**

273

274 **3.1 Endotoxin removal from phage lysates**

275

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

280

281 **3.2 Determination of CD8+ T-cells by flow cytometry**

282

283 The percentage of CD8⁺ T cells within the CD8⁺ lymphocyte population was significantly
284 enhanced in mice injected with λF7 (gpD::AE37) compared to controls groups ($P < 0.05$) (Fig.
285 1).

286

287 **3.3 Antigen-specific cytotoxicity by λF7 (gpD::AE37)**

288 Cytotoxicity assays provide an *in vitro* evaluation of the lytic activity of T cells against tumors
289 [26]. The λF7 (gpD::AE37) phage was significantly effective in generating CTL response. The
290 activity was established significantly at both various effector to target (E/T) ratio (at 2.5/1 and
291 10/1) in comparison with the λF7 and TN buffer groups ($P < 0.0001$) and ($P < 0.001$). This
292 response was antigen specific because the CTL response was not observed against CT26 tumor
293 cells (rHER2/neu negative) (Fig. 2).

294 **3.4 IL-4 and IFN-γ production assays**

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

298

299 **3.5 Prophylactic study**

300

301 10^8 PFU of AE37 displaying phages and λ F7 (100 μL/mouse) were used to vaccinate mice
302 subcutaneously at two week intervals. Two weeks after the third vaccination all groups were
303 challenged with 5×10^5 TUBO cells by injection on right flank and the mice were observed for
304 any touchable tumor on the right flank. Tumor growth curve analysis indicated that the λF7
305 (gpD::AE37) and λF7 groups were the most effective groups in terms of reducing the growth rate
306 of the tumor ($P < 0.0001$) and ($P < 0.001$) in comparison to TN buffer (Fig. 4.A). The
307 prophylactic effects observed in mice model groups are summarized in Table 2 indicating
308 median survival time (MST), time to reach end point (TTE) and tumor growth delay (% TGD)
309 for each mice group.

310 Survival analysis revealed that the λF7 (gpD::AE37) and λF7 group had significantly prolonged
311 MST, TTE and % TGD compared to the TN buffer ($P < 0.01$) (Fig. 4.B).

312

313

314 **3.6 Therapeutic study**

315

316 In the therapeutic evaluation study, 5×10^5 TUBO cells per mice were subcutaneously injected in
317 the right flank of experimental and control animals. After observation of a palpable tumor,
318 vaccination was started for three times with two week intervals. Weights of mice and sizes of
319 tumors were measured regularly until mice became lethargic or size of tumors was reached up to
320 1000 mm^3 . The candidate vaccine formula λF7 (gpD::AE37) decreased the size of tumor

321 significantly ($P < 0.001$) and λ F7 group ($P < 0.05$) in comparison with TN buffer (Fig. 5. A). In
322 the λ F7 (gpD::AE37) vaccination group an increased survival time was observed ($P < 0.05$)
323 which in comparison with λ F7 and TN buffer groups was statistically significant (Fig. 5. B). The
324 therapeutic effects observed in mice model groups are summarized in Table 3 indicating median
325 survival time (MST), time to reach end point (TTE) and tumor growth delay (% TGD) for each
326 mice group.

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330

331 4. Discussion

332

333 The goal of our study was to investigate the immunogenicity and anti-tumor activity of the
334 chimeric λ phage nanoparticles displaying immunogenic AE37 peptide, λ F7 (gpD::AE37) in a
335 TUBO tumor model of BALB/c mice. Cancer peptide vaccines, based on tumor-associated
336 antigens (TAA), can induce cellular and humoral immune response against the tumor or they
337 may cause an enhancement of an endogenous antitumor immunity pre-existing in the host [27].
338 HER2/neu is a receptor belonging to the epidermal growth factor receptor family the
339 overexpression of which has been observed in 18–20% of human breast cancers and linked to a
340 poor prognosis [28].

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

360

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

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

374 The lambda phage vector is about 1000 times more efficient than a plasmid vector to transform a
375 target peptide molecule. It can be converted to a plasmid for the production of foreign peptides
376 and proteins. Moreover, the ease of plaque screening and the efficiency of infection with lambda
377 is evident [38]. In a recent study, we have shown that λ F7 (gpD::E75) particles displaying the
378 E75 peptide (another HER2 derived molecule), can stimulate specific CD8+ T cells *in vitro* [1].
379 Lambda and T7 phage display systems have shown to be able to elicit a B cell response in
380 cancer cells [39]. Recombinant λ phage nanoparticles carrying HBsAg (hepatitis B surface
381 antigen) stimulated specific antibodies production in rabbits and mice [40]. The peptide
382 inoculant λ gfp10-GFP-TAT and hybrid DNA could induce the greatest amplitude of an IFN- γ
383 production in CD1 mice [41]. Phages displaying the melanoma antigen (MAGE₁₆₁₋₁₆₉) produced
384 significant CTL immune responses against a tumor-associated antigen epitope leading to
385 therapeutic and protective effects in a C57BL/6J mice model [42].

386 In this study, we employed λ F7 phage particles displaying the AE37 peptide (AE36, HER2₇₇₆₋₇₉₀
387 linked to LRMK peptide) on λ F7 (gpD::AE37). The immunogenicity and antitumor potential of
388 the λ F7 (gpD::AE37) was investigated using *in vivo* and *in vitro* assays. BALB/c mice were
389 immunized subcutaneously three times with endotoxin-free λ F7 (gpD::AE37) phage
390 nanoparticles. Our data demonstrated that the λ phage could act as an endogenous adjuvant. The
391 bacterial pathogen-associated molecular patterns (PAMPs) probably act as an endogenous
392 adjuvant. The observed immunogenicity of the control phage in the prophylactic and therapeutic
393 settings has been reported consistently in our other projects including one of our recently
394 published studies [43]. The effects in the prophylactic setting may even be higher due to longer
395 exposure time. Higher prophylactic (compared to therapeutic) effects of bacteriophages has been
396 reported in other settings [44]. It has also been reported by many investigators that phage
397 particles are inherently immunogenic and can serve as effective natural adjuvants. This is why
398 phage display vaccines may be effective without adjuvants that are frequently used along with
399 recombinant proteins and synthetic peptides to improve immune response [45]. This inherent
400 immunogenicity is considered a great advantage for the phage vaccines in addition to their many

401 other advantages including their high multivalent display potential, safety profile, and ease of
402 manufacturing and construction [46, 47]. Therefore, the observed immunogenicity by the empty
403 phage particles are expected to be seen. The flow cytometry assays showed that mice
404 immunized with λ F7 (gpD::AE37) nanoparticles could induce a significant CD8⁺ T-cell response
405 compared to control groups. In addition, cytotoxicity assays showed that mice immunized with
406 λ F7 (gpD::AE37) nanoparticles could induce a higher CTL activity compared to λ F7 and buffer
407 groups indicating the crucial role of the repetitive display of AE37 peptide on the surface of λ
408 nanoparticles. *In vivo* studies demonstrated that λ F7 (gpD::AE37) nanoparticles decreased tumor
409 growth and possessed superior anti-tumor activity in both prophylactic and therapeutic assays.
410 Also, it was able to increase the survival time of the TUBO cell line (HER2-over expressing)
411 tumor bearing mice. We also report that λ F7 successfully induced tumor-specific CTL activity
412 immune responses in comparison to the TN buffer. The control λ F7 did not show *in vitro*
413 induction of CD8⁺ T-cell and CTL activity.

414 In summary, the results of this study demonstrates that vaccination with lambda phage
415 nanoparticles expressing AE37 peptide, λ F7 (gpD::AE37) led to the induction of specific CTL
416 immune response *in vitro* and *in vivo*. Moreover, the delivery of AE37 peptide by λ
417 bacteriophage has significantly enhanced the anti-tumor immune function compared to the
418 control groups. In fact, we think that there is a potential for the λ F7 (gpD::AE37) phage
419 nanoparticles for antitumor vaccine development as the phage construct was found to elicit
420 inhibitory effects on the TUBO tumor (HER2/neu overexpressing implantable tumor) and
421 therefore we think that our data merits further investigation. Further studies into the exploitation
422 of the vaccine candidate is underway.

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424

425 5. Conflicts of interest

426

427 The authors declare no conflicts of interest.

428

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430

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435 7. References

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568

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

Designation	Genotype	Source/Reference
Bacterial strains		
BB4	<i>supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA DE(lac)</i> U169	Agilent Technologies, Inc
W3101	F ⁻ , <i>galT22, λ⁻, IN(rrnD-rrnE)1, rph-1</i>	CGSC #4467, Bachmann <i>et al</i> (Bachmann, 1972)
W3101 SupE	F ⁻ , <i>galT22, λ⁻, IN(rrnD-rrnE)1, rph-1 crcA280::Tn10, glnV44(AS)</i>	Nicastro <i>et al</i> (Nicastro, et al., 2013)
Phages		
λF7	<i>λDam15imm21CIts</i>	Mikawa <i>et al</i> (Mikawa, et al., 1996)
λF7 (gpD::AE37)	<i>λDam15imm21CIts</i>	This study
Plasmids		
pPL451 gpD	<i>pM-cl857-pL-cl857-pL- D-tL</i>	Sokolenko <i>et al</i> (Sokolenko, et al., 2012)
pGPD::AE37	<i>pM-cl857-pL-cl857-pL- D::E37-tL</i>	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

ACCEPTED MANUSCRIPT

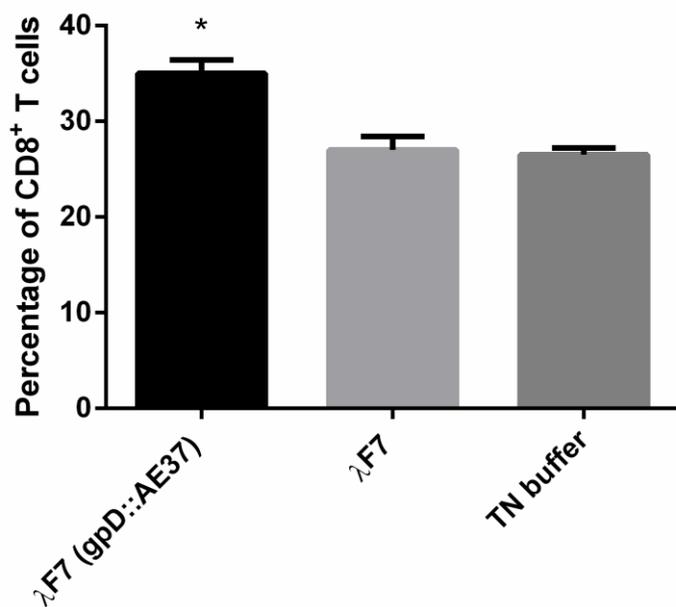


Fig. 1. Flow cytometry assay with splenocytes of immunized mice. Fourteen days after the last immunization, splenocytes were isolated and stimulated *in vitro* with PMA/I for 4 h and stained with a surface CD8. Percentage of CD8+ T cells significantly increased in λF7 (gpD::E37) group compared to λF7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

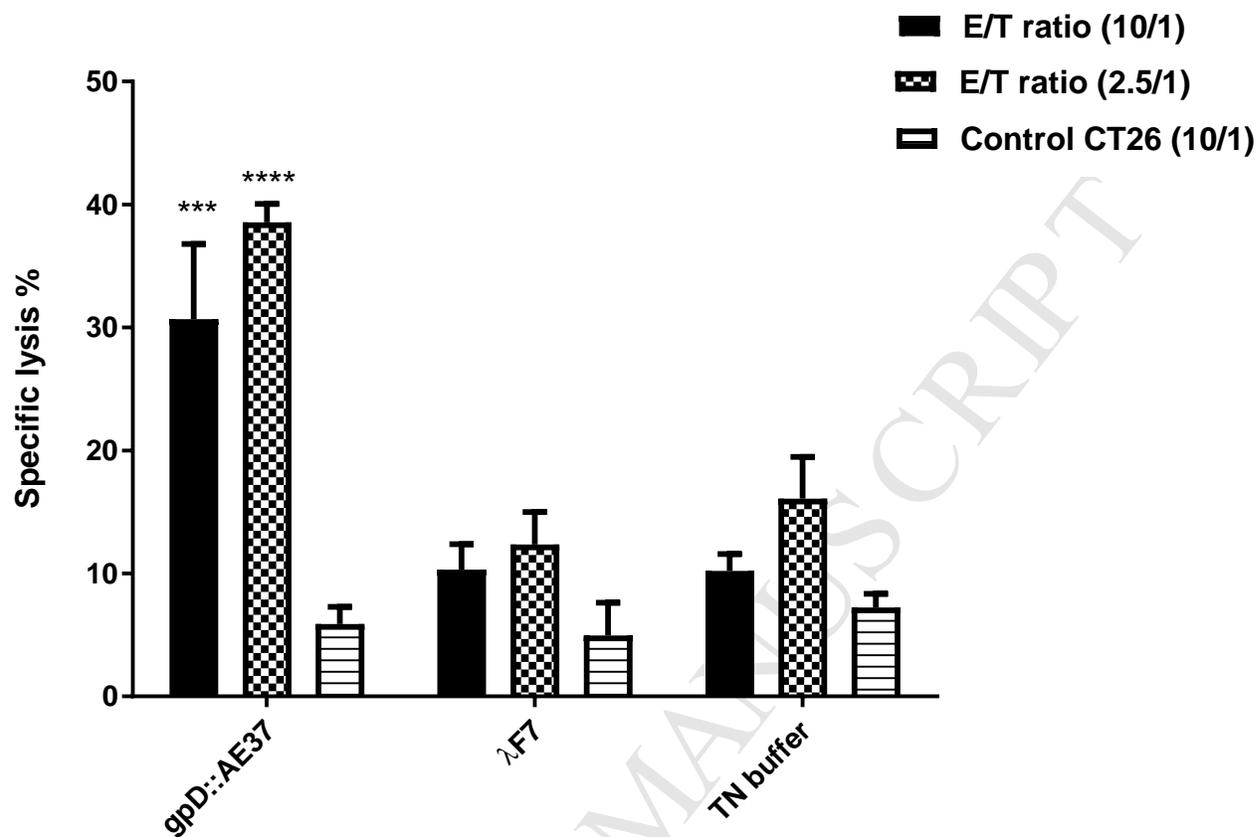


Fig. 2. Antigen - Specific CTL response induced by various formulations at two different ratios of effector to target cells (E/T) was assessed using an *in vitro* CTL activity assay. Splenocytes from the mice (from three mice in each group) were incubated with Calcein AM-loaded rHER2/neu-expressing TUBO 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 \pm SEM ($n = 3$).

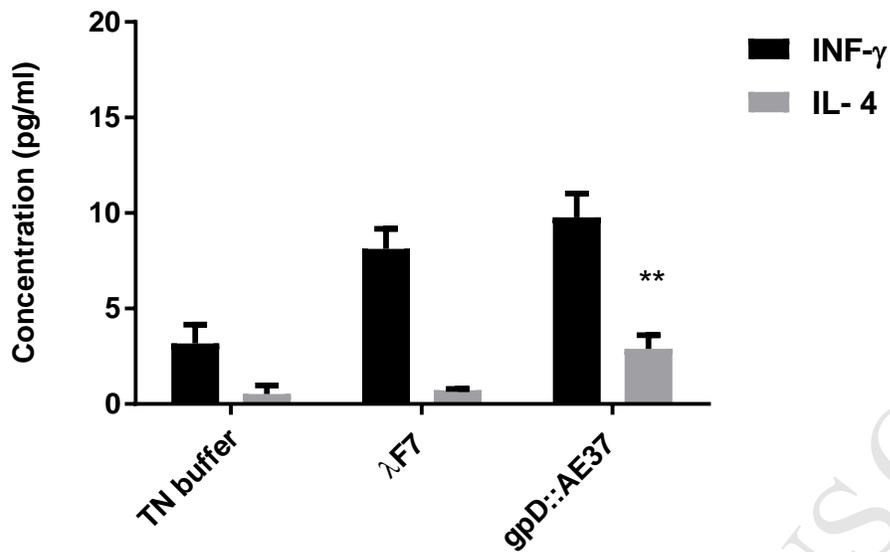


Fig. 3. Secretion of IL-4 and IFN- γ cytokines induced by phages expressing AE37 peptide. BALB/c mice were immunized with λ F7 (gpD::AE37) every 2 weeks for three times. Blood samples were collected 14 days after the last booster and the concentration of IL-4 and IFN- γ cytokines was determined using ELISA. Mice immunized with λ F7 (gpD::AE37) showed higher levels of IL-4 and IFN- γ cytokines compared to λ F7 and buffer groups. Data represent mean \pm SD (n = 3). ** (P < 0.01)

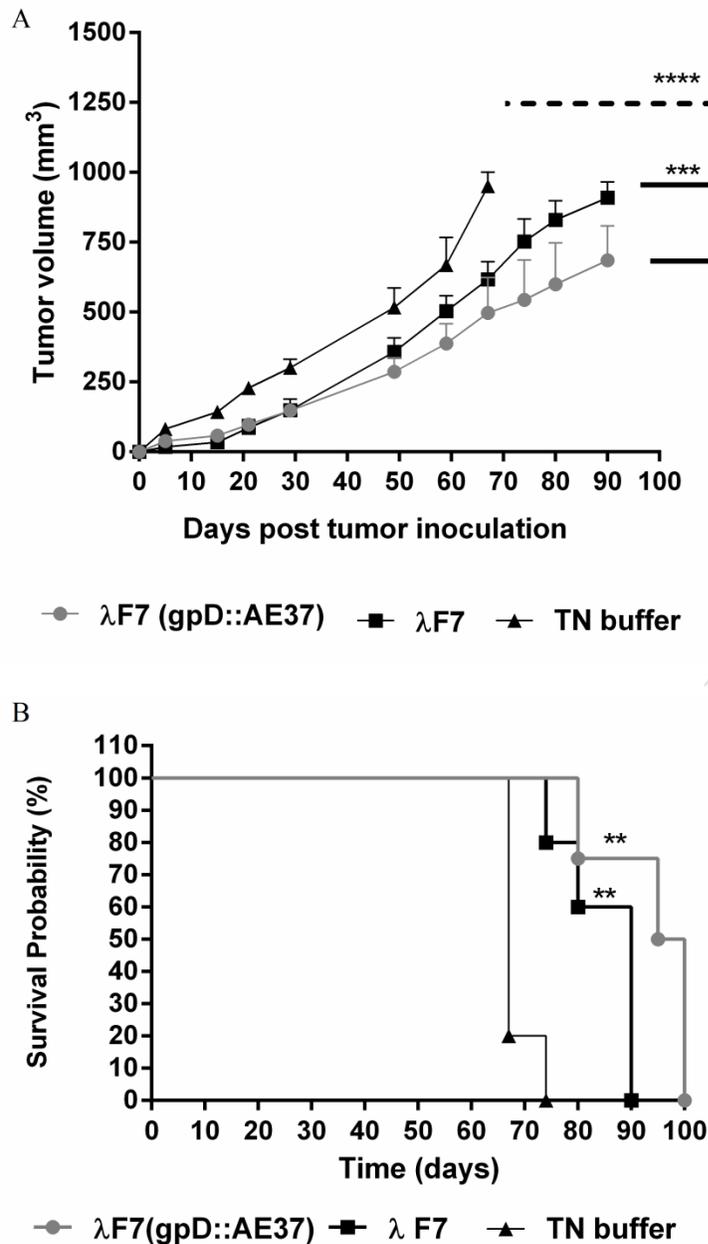


Fig. 4. Protective effects of vaccination with λ F7 (gpD::AE37) phage in BALB/c mice against a TUBO tumor model. Two weeks after the last booster, seven mice in each group were challenged subcutaneously on right flank with 5×10^5 TUBO cells. Mice were observed for tumor growth (A) and survival (B). Tumor size was calculated twice per week, based on the three dimensions. The survival of mice was followed for 100 days. The data indicate mean \pm SEM ($n = 7$). ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$; denotes significant difference from the TN buffer and control groups.

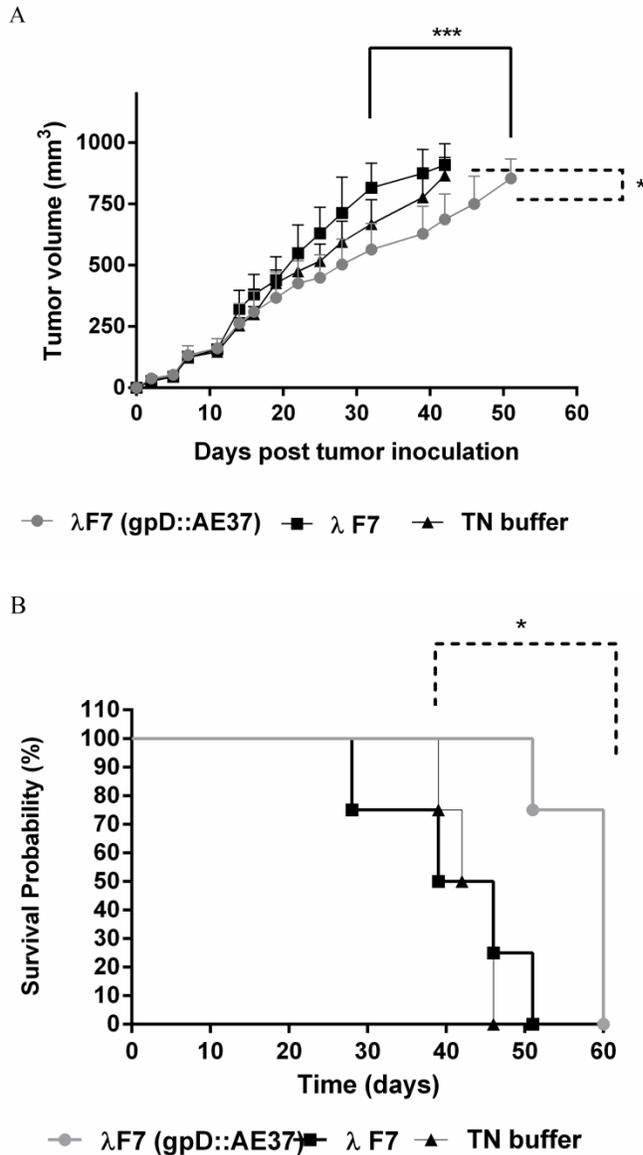


Fig. 5. Therapeutic effects of $\lambda\text{F7 (gpD::AE37)}$ phage nanoparticles against the HER2 expressing TUBO tumor of BALB/c mice. Two weeks after injection of 5×10^5 TUBO cells (seven mice in each group) the mice were administrated with the samples for three times at two week intervals. After 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 \pm 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.