Bacteriophage-mediated therapy of chondrosarcoma by selective delivery of the tumor necrosis factor alpha (TNFα) gene

Aitthiphon Chongchai1,2 | Sajee Waramit2 | Keittisak Suwan2 | Mariam Al-Bahrani2 | Sasimol Udomruk1 | Thanyaluck Phitak1 | Prachya Kongtawelert1 | Peraphan Pothacharoen1 | Amin Hajitou2

1Thailand Excellence Centre for Tissue Engineering and Stem Cells, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
2Cancer Phage Therapy Group, Department of Brain Sciences, Faculty of Medicine, Imperial College London, London, UK

Abstract
Chondrosarcoma is a cartilage-forming bone tumor, well known for intrinsic resistance to chemotherapy and radiotherapy. We have designed a targeted chondrosarcoma gene therapy using a bacteriophage (phage) particle to deliver therapeutic genes. Phage has no tropism for mammalian cells, allowing engineered phage to be targeted to specific cell surface receptors in cancer. We modified the phage capsid to display the RGD4C ligand on the pIII minor coat proteins to specifically bind to αvβ3 or αvβ5 integrin receptors. The endosomal escape peptide, H5WYG, was also displayed on recombinant pVIII major coat proteins to enhance gene delivery. Finally, a human tumor necrosis factor alpha (TNFα) therapeutic transgene expression cassette was incorporated into the phage genome. First, we found that human chondrosarcoma cells (SW1353) have high expression of αvβ3, αvβ5 integrin receptors, and both TNFα receptors. Targeted particle encoding a luciferase reporter gene efficiently and selectively mediated gene delivery to these cells. When SW1353 cells were treated with the targeted particle encoding a TNFα transgene, significant cell killing was evident and was associated with high expression of TNFα and apoptosis-related genes. In vivo, mice with established human chondrosarcoma showed suppression of tumors upon repetitive intravenous administrations of the targeted phage. These data show that our phage-based particle is a promising, selective, and efficient tool for targeted chondrosarcoma therapy.
1 | INTRODUCTION

Chondrosarcoma (CS) is a group of cartilage-forming tumors. It accounts for 20-27% of primary malignant bone neoplasms. The most common anatomical location of this cancer is the pelvis, followed by the proximal femur, proximal humerus, distal femur, and ribs. Most CS patients suffer pain, swelling, and the presence of a mass of variable duration. It is intrinsically resistant to chemotherapy and radiotherapy because of the hyaline cartilaginous matrix, low percentage of dividing cells, slow division rate, and poor vascularization; so far leaving surgical removal as the only therapeutic option. However, in some patients, local recurrence or metastasis occurs. Most of this disease exists as conventional CS (more than 90%) and 5-10% of these are grade 3 with high metastatic potential. Patients with metastatic CS often have a poor prognosis, resulting in death. Clinical management of CS is particularly challenging; therefore, effective therapeutic strategies are urgently needed. Cancer gene therapy is an upcoming approach for cancer treatment. It has been tested clinically in several types of cancer and showed promising outcomes; however, there are limited gene therapy studies in chondrosarcoma. Gene therapy for cancer has been attempted for the past 26 years, but progress has been hindered mostly by challenges faced by eukaryotic viral gene delivery vectors following administration through clinical systemic routes. Although this could be bypassed by local delivery of vectors, but real clinical benefit can only happen with systemic administration, mainly for the treatment of metastatic cancer responsible for more than 90% of cancer patient death.

We have introduced a prokaryotic viral-based approach of systemic gene delivery to target tumors by using filamentous M13 bacteriophage (phage) as a vector to deliver therapeutic genes. Phage has evolved to infect bacteria only and lacks native tropism to eukaryotic cells. Production of a chimeric vector by combination of phage and genetic cis elements, inverted terminal repeats (ITRs), from the human adeno-associated virus (AAV2) is a key strategy of this systemic delivery platform, resulting in a phage capsid which incorporates recombinant AAV genomes. Importantly, the phage capsid was engineered to display the double CDCRGDCFC (RGD4C) ligand that binds the heterodimer αvβ3 integrin cell surface receptor, which is overexpressed on tumor cells and supporting angiogenic vasculature in most tumor types. Overexpression of αvβ3 integrin on human chondrosarcoma cell surface has been reported by Lai et al. Upon binding the αvβ3 integrin receptor and subsequent entry of the phage particles into cells, the AAV transgene expression cassette is released to express the transgene in tumors from a cytomegalovirus, CMV, promoter. Recently, we have further refined the technology through capsid modification by display of the histidine-rich endosomal escape peptide, H5WYG (sequence GLFHAIAHFIHGGWHGLIHGWYG), on recombinant pIII major coat proteins to boost phage escape from the endosomes, subsequently resulting in enhanced gene delivery both in vitro and in vivo (Figure 1). We previously reported sequestration of phage in the endosomal/lysosomal degradative pathway as a major intracellular limitation to phage-based gene delivery vectors and designed a multifunctional phage vector that can simultaneously express (i) the RGD4C targeting ligand on the pIII minor coat proteins, (ii) endosomal escape peptide on recombinant pVIII major coat proteins, and (iii) package a mammalian transgene expression cassette flanked by AAV2 ITRs (Figure 1). Next, we inserted in the vector, a DNA sequence for the human tumor necrosis factor alpha (TNFα) as a candidate therapeutic transgene for targeted destruction of human CS (Figure 1). In the body, TNFα is released via proteolytic cleavage from transmembrane tTNFα by the metalloprotease TNF- alpha converting enzyme (TACE). More specifically, TNFα mainly activates the TNFR1 receptor, which is constitutively expressed by almost any cell type but it has limited signaling capacities on TNFR2 receptor, which is expressed in immune cells including myeloid cells, regulatory T cells, glial cells, and some endothelial cell types. Interestingly, TNFα promotes significant cytotoxicity by activating its receptors and induces apoptosis of many tumor cell types. Moreover, in a safety study in companion dogs, the original version of our vector carrying a human TNFα proved efficient against spontaneous sarcomas in those pet dogs.

Accordingly, in this study, we evaluated the efficacy of our multifunctional phage for the ability to (i) target human CS, (ii) deliver gene expression, and (iii) induce CS destruction, by vector carrying a TNFα DNA sequence, in vitro and in a pre-clinical model of human CS established in mice, upon intravenous administration.

2 | MATERIALS AND METHODS

2.1 | Construction and production of superior bacteriophage-based particle

To construct the bacteriophage-based vector for targeted gene delivery, the M13 phage genome was modified to display RGD4C ligand, tumor homing peptide, on pIII minor coat proteins and the endosome escape peptide H5WYG.
CHONGCHAI et al. (GLFHAIAHFIHGGWHGLIHGWYG) on the recombinant pVIII coat proteins. The vector was further modified to carry a mammalian transgene cassette composed of a cytomegalovirus, CMV promoter, a transgene, and ITR cis elements from AAV2 (Figure 1A). The DNA sequence encoding a secreted version of TNFα was inserted within the ITR-flanked transgene expression cassette under the control of the CMV promoter (Figure 1A). The TNFα transgene expression cassette flanked by AAV ITRs was genetically inserted into the phage genome. B, Electron microscopic images of targeted RGD4C/H5W-Phage-TNFα and control non-targeted H5W-Phage-TNFα filamentous bacteriophage-based particles are shown.

2.2 | Cell culture

Human chondrosarcoma cell line (SW1353) was purchased from American Type Culture Collection (ATCC). Primary human articular chondrocytes (HACs) were isolated from non-osteoarthritic joints taken from normal cartilage of male patients aged 27 years at Maharaj Nakorn Chiang Mai Hospital with informed consent and local ethical committee approval (Ethic approval no. ORT-11-09-16A-14). Cancer cells and the isolated chondrocytes were maintained in a humidified atmosphere of 37°C in a 5% CO₂ and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma, UK) supplemented with 10% fetal bovine serum (FBS, Sigma, UK), penicillin (100 units/mL, Sigma, UK), streptomycin (100 μg/mL, Sigma, UK), and L-glutamine (2 mM, Sigma, UK). Primary human articular chondrocytes were used up to 4 passages.
2.3 | Flow cytometry

SW1353 cells and HACs were harvested using cell dissociation buffer (Life Tech, UK) and washed twice with phosphate-buffered saline (PBS). Cell numbers were adjusted to a concentration of $1 \times 10^6$ cells/mL in cold Fluorescence-activated Cell Sorting (FACS) buffer.

For integrins expression, cells were stained with either heterodimer αvβ3 or αvβ5 antibodies (diluted 1:50 in FACS buffer, R&D systems, UK) for 30 minutes at room temperature in the dark, then washed three times with FACS buffer. Subsequently, cells were stained with AlexaFluor-488 conjugated mouse IgG secondary antibody diluted 1:1000 in 2% BSA in PBS (Thermo Fisher Scientific, UK) for another 30 minutes at room temperature in the dark, then washed three times with FACS buffer. The expression was measured by flow cytometry, and analysis was conducted by FlowJo software (BD Biosciences, US). Cells stained with the secondary antibody alone were used as control.

For death receptors expression, cells were stained with primary antibodies against either TNFR1 or TNFR2 (diluted 1:50 in FACS buffer, R&D systems, UK) for 30 minutes at room temperature, then washed three times with FACS buffer. The cells were counterstained with AlexaFluor-488 conjugated secondary antibody (diluted 1:750 in 2% BSA/PBS, Invitrogen, Thermo Fisher Scientific, UK) for 30 minutes at room temperature, then washed three times with the FACS buffer. Expression was measured by flow cytometry, and analysis was conducted by FlowJo software (BD Biosciences, US). Cells stained with the secondary antibody alone were used as control.

2.4 | In vitro cell transduction by bacteriophage-based vectors

SW1353 cells and HACs were seeded into culture plates and grown for 24 hours to reach 60%-70% confluence. Prior to transduction, culture medium was replaced with serum-free medium for 2 hours. Cells were transduced with phage-based particles diluted in serum-free medium and incubated in a CO₂ incubator at 37°C. Next, cells or cultured media were collected and subsequently analyzed.

2.5 | RNA purification and RT-qPCR

Cells were collected, and RNA was extracted using an RNA extraction kit (PureLink RNA Mini Kit, Thermo Fisher Scientific, UK) following the manufacturer’s protocol. A total RNA (1 µg) was converted to cDNA using a High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Thermo Fisher Scientific, UK) following the manufacturer’s protocol. qPCR was performed to quantify sTNFα and expression of apoptotic related genes by using PowerUp SYBR Green Master Mix.

### Table 1 Primer sequences for RT-qPCR

| Gene (human) | Primers (5′ → 3′) |
|-------------|-------------------|
| TNFα        | Forward: GGAGTAGATGAGGTACAGGCC <br>Reverse: CGTCAGATCAGTTCCTCGAGC |
| Caspase 3    | Forward: TGGTCATCCAGTCGTTTG <br>Reverse: CATCTGTTGTCATTGACG |
| Caspase 7    | Forward: CCAATAAGAGATTGACAGCC <br>Reverse: GCATCTGTTGTCATTGACG |
| Caspase 8    | Forward: GGCTCCTCAGAAGTCCTCTCTCAGAACA |
| Caspase 9    | Forward: CAACTAAACAGGCAAGCAGC <br>Reverse: ACCCTAAGAATCTCCCAAGAAG |
| BID          | Forward: GTCTCACCTGTAGTGAGT |
| BAX          | Forward: CCGTGTCACCAAGTGTCGGAAG <br>Reverse: CACCCCTGGTCCTGGATCCAGC |
| PARP         | Forward: AAGGCGAATGCGCAGAGGT <br>Reverse: GACCTTCAGGACCATCAGTCA |
| GAPDH        | Forward: CCCCTTCATTGACCTCAACTAC <br>Reverse: GATGACAAATCCTCCGGTC |

Abbreviations: Bax-2-associated X protein; BID, Bcl-2–associated X protein; BID, BH3 interacting-domain death agonist; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase; TNFα, tumor necrosis factor alpha.

(Termo Fisher Scientific, UK). The primers were purchased from Life Technologies, UK, primer sequences are listed in Table 1. The level of gene expression was relative to that of the control group according to $2^{-\Delta\Delta CT}$ calculation. GAPDH (the house-keeping gene) was included as internal control.

2.6 | Cell death assays

2.6.1 | The sulforhodamine B (SRB) assay

Transduced cells were washed with PBS, then fixed with cold trichloroacetic acid (TCA) overnight at 4°C. Next, cells were washed under slow running tap water for 4 minutes and left air-dry. Next, the cells were stained with 0.4% SRB solution (Sigma, UK) for 1 hour on an orbital shaker and then washed with 1% acetic acid and left air-dry. Finally, the stained cells were dissolved in 10 mM Tris pH 10.5 and left on the shaker. Spectrophotometric measurement at the absorbance 490 nm was performed using an automated microplate reader.

2.6.2 | CellTiter-Glo Luminescent Cell Viability Assay

CellTiter-Glo Reagent (Promega, UK) was added to the culture well containing transduced cells at equal volume of culture
media and then mixed for two minutes on a shaker to induce cell lysis. Next, the mixture was incubated at room temperature for 10 minutes to stabilize the luminescent signal and transferred to opaque-walled multi-well plates. The signal was detected using GloMax Navigator Microplate Luminometer (Promega, UK).

2.6.3 | Crystal violet staining

Transduced cells in 96-well plates were washed twice in a gentle stream of tap water and left air-dry. Next, 0.5% crystal violet staining solution (Sigma, UK) was added to the culture and incubated for 20 minutes on a bench rocker. The cells were then washed four times in a stream of tap water and left air-dry. Distilled water was added to each well, and viable cells were observed under the microscope.

2.7 | ELISA for TNFα quantification

The ELISA plate was coated with diluted Human TNFα ELISA MAX Capture Antibody solution and incubated overnight at 4°C. Then, the plate was washed 4 times with 0.05% Tween/PBS and blocked by adding 1% BSA in PBS at room temperature for 1 hour with shaking on a plate shaker. The plate was washed 4 times with 0.05% Tween/PBS before adding diluted human TNFα standards or samples and then incubated at room temperature for 2 hours with shaking. After this step, the plate was washed 4 times with 0.05% Tween/PBS then added diluted human TNFα ELISA MAX Detection Antibody solution and incubated at room temperature for 1 hour with shaking. Next, the plate was washed 4 times with 0.05% Tween/PBS then added diluted Avidin-HRP solution and incubated at room temperature for 30 minutes with shaking. The plate was then washed 5 times with 0.05% Tween/PBS then added TMB substrate solution and incubated in the dark for 15-30 minutes. Finally, the reaction was stopped by adding 2N H₂SO₄ and absorbance measured at 450 nm.

2.8 | Lentivirus production and generation of SW1353-GFP-Luc cell line

The 293T17 cells were seeded on T-175 flasks in complete medium (10% FBS/DMEM supplemented with 1% L-glutamine, 1% sodium pyruvate, and 1% NEAA) for 24 hours. The culture medium was replaced 2 hours prior to transfection. We prepared the plasmid DNA for lentivirus production by combining expression vector (pLIV-GFP-Luc) with packaging vector (pspAX2 and pMD2.G) in dd H₂O, then gently added CaCl₂ and bubbled through HEPES-buffered saline. The solution was mixed and incubated at room temperature for 30 minutes then mixed again prior to adding dropwise to cells. The cells were incubated at 37°C, 3% CO₂ for 4 hours then washed twice with PBS. Next, DMEM complete medium containing sodium butyrate was added to the culture and incubated at 37°C, 3% CO₂ for overnight. We collected 24 hours post-transfection medium and replaced with DMEM complete medium containing sodium butyrate and incubated for another 24 hours before the final collection (48 hours post-transfection medium). The lentivirus containing media were combined, spun at 1,500 rpm for 10 minutes, placed into a new tube, filtered through 0.45 µm low protein binding PVDF filter, and stored in the fridge. The SW1353 cells were seeded on T-175 flasks in complete medium (10% FBS/DMEM supplemented with 1% L-glutamine, penicillin, and streptomycin) in a humidified atmosphere of 37°C, 5% CO₂ for 24 hours. The cells were incubated with lentivirus containing medium for 24 hours then replaced with DMEM complete medium daily until the expression of GFP and Luciferase transgene were observed. The cells were sorted for GFP expression by FACS. After this step, GFP-positive cells were cultured, expanded, and monitored for expression of GFP and Luciferase transgenes before cell implantation into the mice.

2.9 | In vivo experiments in tumor-bearing mice

All procedures were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB committee) at Imperial College London, performed in accordance with the Institutional and Home Office Guidelines, and under a granted Home Office-issued project license. Athymic mice (BALB/c nu/nu 6 weeks old) were acquired from Charles River, United Kingdom. Human chondrosarcoma cells were subcutaneously established in athymic mice using the SW1353 cells stably expressing GFP and firefly luciferase (Luc) reporter genes (SW1353-GFP-Luc) at 2 × 10⁶ cells per mouse. Tumor-bearing mice (n = 4) were intravenously injected with targeted (RGD4C) or non-targeted phage particles carrying the sTNFα gene at a dose of 5 × 10¹⁰ TU per mouse twice a week. We monitored the animal body weights and luciferase expression of mice in each group. Tumor growth was monitored twice a week by BLI of luciferase. Mice were anesthetized, administered 100 mg/kg of D-luciferin (Gold Biotechnology), and then imaged by the In Vivo Imaging System (IVIS 100; Caliper Life Sciences). A region of interest (ROI) was determined manually over the tumors to estimate the signal intensities reported as total photon counts per second per cm² (p/sec/cm²/sr). At the end of therapy, mice were sacrificed by terminal perfusion through the heart.
2.10 | Negative staining for transmission electron microscopy of the phage particles

We glow discharged on formvar carbon-coated 200 mesh grids at 10 mA for 2 minutes. Phage particles were applied on the grids and incubated for 1 minute then removed by blotting on absorbent paper. Next, the grids were washed with filtered deionized water and blotted on absorbent paper then floated on drop of filtered deionized water for 3 minutes and blotted on absorbent paper. The grids were then floated on drop of 1% glutaraldehyde for 5 minutes and blotted on absorbent paper. The particles were negatively stained by applying 1% uranyl acetate to the grid for 4 minutes. The grids were then blotted on absorbent paper, and images were finally taken using a transmission electron microscope (JEOL JEM-2010, Japan) and analyzed by using ImageJ software.

2.11 | Statistical analysis

All data were represented as mean ± SEM. For statistical analyses, we used independent t test, one-way ANOVA, Tukey’s HSD post hoc test, Mann-Whitney U test, and Kruskal-Wallis test. Statistical significance was expressed as P values of <.05, .01 and .001. All statistical analyzes were invented using SPSS software.

3 | RESULTS

3.1 | Human chondrosarcoma cells express αvβ3 and αvβ5 integrin receptors for the tumor targeting RGD4C/H5W-Phage

The multifunctional phage particle, RGD4C/H5W-Phage, used in this study, displays a double-cyclic RGD4C ligand on the pIII minor capsid proteins (Figure 1A) to serve as a tumor targeting ligand; it mainly binds to αvβ3 integrin receptor and to a lesser extend to αvβ5, both present on the surface of cancer cells.15,25,26 Moreover, the multifunctional RGD4C/H5W-Phage expresses the endosomal escape peptide, H5WYG, on the recombinant pVIII coat proteins and carries an AAV2 ITR-flanked transgene cassette inserted within the phage genome (Figure 1A). Transmission electronic microscopic (TEM) analysis of vectors confirmed the filamentous morphology and particle size of the multifunctional RGD4C/H5W-Phage and non-targeted H5W-Phage (Figure 1B). Next, we investigated expression of the integrin receptors on the human chondrosarcoma cell line (SW1353) and compared to normal primary human articular chondrocytes (HACs). Both cells were stained with antibodies against heterodimer αvβ3 or αvβ5 integrins and analyzed by flow cytometry. As shown in Figure 2A,C, SW1353 cells were approximately 38 and 39 percent positive for αvβ3 and αvβ5 integrin expression, respectively. Interestingly, as shown in Figure 2B,D. HACs barely express the αvβ3 and αvβ5 integrins, which were only 1.5 and 2 percent, respectively. Therefore, we concluded that SW1353 cells can be used as a human CS model for our tumor-targeted phage-based particle. Importantly, the very low expression profile of both integrins on the normal HACs ensures that normal chondrocytes will be spared from off-target binding of the vector.

3.2 | Tumor-targeted phage vector efficiently targets and transduces human chondrosarcoma cells, but not normal primary human chondrocytes

To evaluate transduction efficacy of the multifunctional phage vector for CS cells, we generated a phage-based particle carrying a secreted luciferase reporter gene, Lucia. SW1353 cells and HACs were treated with two types of vectors which are targeted RGD4C/H5W-Phage-Lucia or non-targeted H5W-Phage-Lucia which served as a control particle for tumor targeting. Different doses of vectors varied from 0.5 × 10^6 to 1 × 10^6 TU per cell were used. We measured Lucia activity at days 3, 4, 5, 6, and 7 post-transduction. The data showed that SW1353 cells treated with RGD4C/H5W-Phage-Lucia vector had a significant increase of Lucia expression, in dose- and time-dependent manners (Figure 3A). Interestingly, no expression of Lucia was detected in SW1353 cells treated with the non-targeted H5W-Phage-Lucia particle. These results confirm that gene delivery by RGD4C/H5W-Phage-Lucia is selective and mediated by the tumor targeting RGD4C ligand. Importantly, similar experiments performed on normal primary human articular chondrocytes did not show any Lucia expression mediated by the targeted or non-targeted vectors (Figure 3B). These results indicate that our multifunctional hybrid RGD4C/H5W-Phage particle selectively and efficiently mediates gene delivery to human chondrosarcoma cells while sparing the healthy normal chondrocytes.

3.3 | Expression of TNFα receptors, TNFR1 and TNFR2, on chondrosarcoma cells

TNFα, well-known for its tumor cytotoxicity, induces apoptotic cell death through binding to two transmembrane receptors, TNFR1 and TNFR2. Interestingly, TNFR1 is constitutively expressed on most cells compared to TNFR2 expression which is restricted to some cell types only.27 Specifically, TNFα mainly activates TNFR1 but has limited signaling capacities on TNFR2.27,28 Targeting tumor cells requires TNFR1 or TNFR2 expression on their cell surface to mediate cell death by TNFα production. We sought to evaluate expression of these receptors by staining SW1353 tumor
cells with specific antibodies against TNFR1 or TNFR2 receptors. Cytometric analysis of data in Figure 4A,C shows that approximately 35% and 11% percent of SW1353 cells expresses TNFR1 and TNFR2, respectively. Next, for comparison, we measured the expressions of these two receptors on normal primary human chondrocytes. Significantly lower expression of both receptors was detected in normal chondrocytes, with ~10% and 4% of cells express TNFR1 (Figure 4B) and TNFR2 (Figure 4D), respectively. Altogether, these data suggest that TNFα is a potential therapeutic agent candidate for chondrosarcoma. Moreover, low level expression of TNFR1 and TNFR2 receptors on normal chondrocytes should further increase the safety of RGD4C/H5W-Phage-TNFα gene therapy since locoregional TNFα secretion from transduced tumor cells should trigger cell death in chondrosarcoma, while leaving normal chondrocytes unharmed.

3.4 | Tumor-targeted phage vector carrying a soluble sTNFα sequence, but not transmembrane tTNFα, mediates chondrosarcoma cell death without harming the normal chondrocytes

After confirming the potential of the multifunctional RGD4C/H5W-Phage to target gene delivery to human CS and expression of TNFR1 and TNFR2 receptors of TNFα on CS, we subsequently constructed and produced multifunctional particles expressing TNFα. Thus, we designed RGD4C/H5W-Phage-sTNFα vector carrying a sequence for the secreted TNFα. Moreover, we constructed phage particles carrying the transmembrane tTNFα DNA sequence for comparison side-by-side with vector carrying sTNFα to select for the most suitable TNFα version to test in further investigation including in vivo experiments. Non-targeted particles, H5W-Phage-sTNFα and H5W-Phage-tTNFα, without the tumor targeting RGD4C motif were also designed and produced as negative controls for tumor targeting. Next, we applied increasing vector doses and measured cell viability at day 5 post-vector transduction. We found that there was no significant difference in CS viability between RGD4C/H5W-Phage-tTNFα and its corresponding control non-targeted H5W-Phage-tTNFα (Figure 5A). Importantly, treatment of CS with RGD4C/H5W-Phage-sTNFα, 0.5 × 10^6 TU/cell, resulted in ~26% cell death which was significantly more pronounced at 1 × 10^6 TU/cell reaching 50% cell death and saturation since cell death plateaued at 50% regardless of the vector dose tested (Figure 5B). Importantly, there was no cell death upon addition of non-targeted H5W-Phage-sTNFα, in all conditions tested (Figure 5B). Therefore, we selected the RGD4C/H5W-Phage-sTNFα at 1 × 10^6 TU/cell as the biotherapeutic
treatment to investigate in further in vitro experiments and in vivo studies. First, microscopic analysis of cells at day 5 post-treatment, revealed clear destruction of CS cells by 1 × 10^6 TU/cell of RGD4C/H5W-Phage-sTNFα whereas cells from control (untreated) and non-targeted H5W-Phage-sTNFα group remained morphologically normal (Figure 5C). Moreover, quantification of secreted sTNFα by ELISA at day 4 post-transduction yielded a concentration of 106 pg/mL (Figure 5D) which was significantly high, whereas sTNFα production from cells treated with the control H5W-Phage-sTNFα was similar to that of untreated cells. Additionally, these data were confirmed by the substantial elevation of sTNFα mRNA expression quantified by RT-qPCR in CS cells treated with RGD4C/H5W-Phage-sTNFα as compared to non-targeted vector (Figure 5E).

Next, to further validate the anti-tumor effect of RGD4C/H5W-Phage-sTNFα against human CS cells, we measured expression of the caspases 3, 7, 8, and 9 as well as pro-apoptotic genes (BID, BAX, and PARP). A significantly higher gene expression of all these genes was found in cells treated with RGD4C/H5W-Phage-sTNFα as compared to the control non-targeted H5W-Phage-sTNFα treated group (Figure 6). Previous studies reported that exposure of cells to sTNFα can induce the expression of pro-apoptotic genes.

Finally, to confirm the safety of our RGD4C/H5W-Phage-sTNFα particle for normal primary human articular chondrocytes, we treated these cells with high doses of RGD4C/H5W-Phage-sTNFα ranging from 0.5 × 10^6 to 2 × 10^6 TU/cell. Groups of untreated cells or cells incubated with non-targeted H5W-Phage-sTNFα were included in the experiment as controls. No cell death or morphological changes were observed in any of the experimental groups (Figure 7A,B). Altogether, these data suggest that RGD4C/H5W-Phage-sTNFα is a potential therapeutic particle to use for targeted and safe treatment of human chondrosarcoma without harming the normal chondrocytes.

3.5 | Systemic delivery of tumor-targeted RGD4C/H5W-Phage-sTNFα suppressed tumor progression in a pre-clinical model of human chondrosarcoma

Next, we evaluated therapeutic efficacy of the systemic administration of RGD4C/H5W-Phage-sTNFα in BALB/c
immunodeficient mice bearing SW1353-derived xenografts established subcutaneously by implantation of SW1353 cells stably expressing the firefly luciferase, *Luc*, reporter imaging gene. *Luc* expression in tumors, luminescence, was monitored and evaluated by bioluminescent imaging (BLI) which is a simple way to detect tumors and to monitor tumor growth, viability, and their response to the treatment.15,16,30,31 Tumor-bearing mice were intravenously injected with targeted RGD4C/H5W-Phage-\(\text{sTNF}\alpha\) or non-targeted H5W-Phage-\(\text{sTNF}\alpha\) vector at a dose of \(5 \times 10^{10}\) TU per mouse, twice a week for the duration of the experiments. At day 14 post-vector treatment, the SW1353-derived tumors grew in size relative to day 0, before treatment initiation, in groups of mice administered with the control non-targeted H5W-Phage-\(\text{sTNF}\alpha\) (Figure 8A,B). Moreover, the luminescence within tumors increased rapidly in H5W-Phage-\(\text{sTNF}\alpha\) group to reach approximately 4-fold higher than the tumor luminescence recorded at day 0 (Figure 8C).

Remarkably, in sharp contrast, at day 14 post-administration of the targeted RGD4C/H5W-Phage-\(\text{sTNF}\alpha\) particle, limited tumor luciferase signals were detected in mice as compared to day 0, showing not only a lack of tumor growth but a substantial suppression of tumor size and tumor viability (Figure 8A-C).

To confirm the RGD4C/H5W-Phage-\(\text{sTNF}\alpha\) safety, we monitored animal weights throughout the duration of the experiment and found that there was no weight loss noticed in the animals during the course of treatment (Figure 8D), and any weight loss, detected by the end of the experiment, was solely related to the tumor burden.

4 | DISCUSSION

In this study, we applied a phage-derived vector as a platform technology to target the delivery of TNF\(\alpha\) transgene to chondrosarcoma, as a novel biotherapeutic strategy to investigate since current chondrosarcoma treatment is limited to surgery. Phage-based vectors are superior to other common eukaryotic viral vectors, in terms of production, safety, genetic modification, cost, and stability. Bacteriophage naturally does not infect mammalian cells, but the phage capsid can be genetically engineered to display a specific ligand that binds a receptor expressed on the target cells or tissue. Hence, we modified the capsid of the M13 filamentous phage to display a double-cyclic RGD4C peptide ligand on its pIII minor coat proteins to serve as targeting ligand to target the \(\alpha\beta3\) or \(\alpha\beta5\) integrin receptors specifically overexpressed on various types of cancer. Then, we confirmed high expression of \(\alpha\beta3\) and \(\alpha\beta5\) integrins on the surface of human chondrosarcoma cells, and their absence on normal primary human chondrocytes. Targeting \(\alpha\beta3\) integrin in human cancer with RGD4C was previously reported in cancer patients.32 Interestingly, the present study confirms that the RGD4C-Phage can target gene delivery to \(\alpha\beta3\) or \(\alpha\beta5\) integrins in chondrosarcoma. Moreover, the RGD4C-Phage was further refined to display...
the H5WYG, an histidylated fusogenic peptide with endosomal buffering capacity, on recombinant pVIII major coat proteins yielding a multifunctional phage with strong endosomal escape ability and advanced gene delivery, as we previously reported. Using vector carrying a reporter gene, we demonstrated selective gene delivery to chondrosarcoma in dose-dependent manner without any detectable gene expression mediated by the non-targeted vector lacking the RGD4C ligands. Also, no gene delivery was detected in normal primary human chondrocytes treated with the tumor-targeted RGD4C/H5W-Phage. These findings highlight that the multifunctional phage is a suitable delivery system to use in targeted systemic delivery of therapeutic genes to chondrosarcoma.

Next, as therapeutic gene, we designed a vector carrying the TNFα which has long been used in gene therapy against cancer. TNFα signaling mediates through the binding to two cell surface receptors including TNFR1 and TNFR2. We detected high expression of TNFR1 and slight expression of TNFR2 on human chondrosarcoma cell surface. However, expressions of these two TNFR were lower in normal primary human articular chondrocytes. Indeed, TNFR1 is expressed on many types of tumor cells and tumor endothelial cells while TNFR2 is expressed on various immune cells. Also, TNFR1 expression was previously reported to be significantly upregulated in human breast cancer tissues and breast cancer cell lines when compared to normal non-tumor
Moreover, there is a large body of studies supporting that TNFα activates apoptosis through TNFR1 but stimulates pro-survival signaling through TNFR2. In addition, TNFR1 interacts with both soluble and transmembrane TNFα but TNFR2 mainly interacts with the transmembrane TNF-α. Therefore, we applied TNFα as a transgene to mediate cell death in CS targeted gene therapy and found that vector carrying the soluble form of TNFα (sTNFα) induces CS cell destruction while no cell damage was induced by vector carrying the membrane bound form of TNFα (tTNFα). These
findings were associated with the high secretion of sTNFα from tumor cells treated with RGD4C/H5W-Phage-sTNFα. These data are also consistent with the upregulation of apoptotic related genes (caspases 3, 7, 8, 9, and BID, BAX, PARP) in RGD4C/H5W-Phage-sTNFα treated cells. Importantly, consistent with our gene delivery experiments, normal primary human articular chondrocytes treated with RGD4C/H5W-Phage-sTNFα remained unharmed proving that cell death induced by the multifunctional RGD4C/H5W-Phage-sTNFα particle is selective to cancer cells and preserves its safety advantage for normal cells as previously reported.18

In vivo, serial systemic administrations of RGD4C/H5W-Phage-sTNFα particle to immunodeficient mice with established CS xenografts resulted in complete elimination of tumor growth and eradication of the tumor size and tumor viability. These in vivo data merit further discussion. Our multifunctional phage particle shows potential for targeted therapy against CS by (i) targeting integrin receptors on CS, (ii) carrying an endosomal escape peptide, and (iii) delivering a sTNFα transgene expression cassette flanked by AAV2 ITRs. Moreover, our vector can be delivered via non-invasive systemic clinical routes, a route that we and collaborators have proven efficient in various pre-clinical models of human cancer including melanoma, breast, prostate, brain, and pancreatic cancer.16,34-38 Furthermore, we previously reported the anti-tumor efficacy of intravenous delivery of our original phage vector in a rat model of human soft tissue sarcoma.34

**FIGURE 8** In vivo treatment of chondrosarcoma SW1353-bearing mice with intravenous administrations of RGD4C/H5W-Phage-sTNFα. Human chondrosarcoma SW1353 cells labeled with a luciferase reporter gene were implanted into immunodeficient mice (BALB/c nu/nu) to establish subcutaneous xenografts. Tumor-bearing mice (n = 4) were intravenously injected with RGD4C/H5W-Phage-sTNFα or non-targeted H5W-Phage-sTNFα vector at a dose of $5 \times 10^{10}$ TU per mouse, twice a week for the duration of the experiment. A, Representative tumor-bearing mice imaged using the In Vivo Bioluminescent Imaging System at day 0, before treatment initiation, and day 14, post-vector administration. B, Average tumor volumes progression in each experimental group. Pre-treatment day 0 was set at 100%. C, The luminescence values of tumors shown as fold change between pre-treatment day 0 and post-vector treatment day 14. D, Average weights of SW1353 tumor-bearing mice, from all experimental groups, measured on days 6, 10, and 14 post-vector administration. *$P < .05$
Also, we and collaborators have previously reported specificity and safety of the first generation of RGD4C/Phage carrying various genes in rodents and pet dogs with natural tumors. To date in all the pre-clinical models tested, biodistribution of gene delivery by the RGD4C/Phage generated gene expression in tumors exclusively without any detectable expression in the healthy tissues.\(^{26,30,31,34-38}\) Moreover, importantly, it is noteworthy to mention that the vector used in this study is an improved version of a tumor-targeted phage carrying TNFα, whose safety was already completed in domesticated dogs with spontaneous cancers.\(^{23}\) In that study, intravenous injections of vector to tumor-bearing dogs resulted in specific tumor homing of the targeted vector particles and subsequently in selective TNFα expression in the tumor tissue with no detectable TNFα in major healthy tissues. Next, dose escalation and repeated vector dosing proved safe and resulted in complete tumor eradication in dogs with aggressive fibrosarcoma.\(^{23}\) Finally, we recently reported biodistribution of the RGD4C/H5W-Phage, used in this study, using bioluminescent imaging of Luc and confirmed specific delivery of the Luc reporter gene expression in tumors with no detectable expression in healthy tissues.\(^{18}\) Altogether, our present and previous studies suggest that the improved version of vector, RGD4C/H5W-Phage-\(s\)TNFα, can be applied to treat CS in a safe and efficient way paving the way toward clinical trials in patients with CS.

The RGD4C ligand, used in this study for tumor targeting, binds mainly to \(\alpha v\beta 3\), but also to a lesser level to the \(\alpha v\beta 5\) heterodimer. Various integrin heterodimers can be found in a wide variety of human cells; however, both \(\alpha v\beta 3\) and \(\alpha v\beta 5\) are highly restricted and typically overexpressed on cancer cells and tumor vasculature.\(^{39,40}\) In Human biopsies, the \(\alpha v\beta 3\) integrin is widely expressed on blood vessels of human tumor biopsy samples but not on vessels of biopsies from normal tissues. Recently, we reported that a panel of normal human primary cells from different histological origins do not express or have very low expression of the \(\alpha v\beta 3\) and \(\alpha v\beta 5\) integrin receptors of RGD4C.\(^{26}\) Notably, this very low integrin profile did not translate into gene delivery to normal cells by RGD4C/phage vectors.

Additionally, the M13 phage (parent of our vector) displaying a peptide library was administered intravenously to cancer patients,\(^{31}\) with no serious clinical side effects observed with serial vector administrations. Indeed, phage-based vectors have a historic safety profile being safely administered to human over many years to treat infectious diseases. Moreover, repeated administrations of our vectors in immunocompetent animals can be safely performed to sustain a therapeutic response, despite IgGs against the phage capsid.\(^{16,23,26}\)

In conclusion, these studies aim to develop targeted gene therapy as a novel treatment approach of chondrosarcoma to overcome limited current procedures. Here, superior bacteriophage-based vector showed a promising selective and efficient tool for chondrosarcoma gene therapy. Importantly, native bacteriophage cannot infect eukaryotic cells and it is safe to use as antibacterial food additives proved by Food and Drug Administration. Therefore, this platform might be a novel therapeutic method for chondrosarcoma.

**ACKNOWLEDGMENTS**

We thank George Smith for reagents. We also thank Paladd Asavarut, Grace Chu, and Kaoutar Bentayebi at Imperial College London, for the technical assistance, and the Department of Brain Sciences for the equipment.

**CONFLICT OF INTEREST**

S. Waramit, K. Suwan, M. Al-Bahrani, and A. Hajitou are inventors on a patent application describing the vector constructs reported here and will be entitled to royalties if licensing or commercialization occurs. The remaining authors declare no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

K. Suwan, P. Kongtawelert, P. Pothacharoen, T. Phitak, and A. Hajitou designed research; A. Chongchai, S. Waramit, M. Al-Bahrani, S. Udomruk, K. Suwan, and A. Hajitou performed experiments; A. Hajitou, K. Suwan, P. Kongtawelert, and P. Pothacharoen contributed new reagents and analytic tools; A. Chongchai, K. Suwan, S. Waramit, and A. Hajitou analyzed data; A. Chongchai, K. Suwan, S. Waramit P. Pothacharoen, and A. Hajitou wrote the paper. All authors critically revised and approved the final manuscript.

**ORCID**

Keittissak Suwan \(\text{https://orcid.org/0000-0001-6542-5841}\)
Amin Hajitou \(\text{https://orcid.org/0000-0003-1119-5686}\)

**REFERENCES**

1. Ye C, Luo Z, Zeng J, Dai M. Chondrosarcoma of the patella: a case report. *Medicine (Baltimore)*. 2017;96(37):e8049.
2. Murphey MD, Walker EA, Wilson AJ, Kransdorf MJ, Temple HT, Gannon FH. From the archives of the AFIP: imaging of primary chondrosarcoma: radiologic-pathologic correlation. *Radiographics*. 2003;23(5):1245-1278.
3. Van Oosterom AT, Dirix LY. Chondrosarcoma and other rare bone sarcomas. *Curr Opin Oncol*. 1990;2(3):495-499.
4. Gelderblom H, Hogenoord PCW, Dijkstra SD, et al. The clinical approach towards chondrosarcoma. *Oncologist*. 2008;13(3):320-329.
5. Chow WA. Chondrosarcoma: biology, genetics, and epigenetics. *F1000Res*. 2018;7:1826.
6. Wang Z, Chen G, Chen X, et al. Predictors of the survival of patients with chondrosarcoma of bone and metastatic disease at diagnosis. *J Cancer*. 2019;10(11):2457-2463.
7. Ayen A, Jimenez Martinez Y, Marchal JA, Bouliazi H. Recent progress in gene therapy for ovarian cancer. *Int J Mol Sci*. 2018;19(7):1930.
8. Kamimura K, Yokoo T, Abe H, et al. Effect of diphtheria toxin-based gene therapy for hepatocellular carcinoma. *Cancers (Basel).* 2020;12(2):472.

9. Sun W, Shi Q, Zhang H, et al. Advances in the techniques and methodologies of cancer gene therapy. *Discov Med.* 2019;27(146):45-55.

10. Libutti SK. Recording 25 years of progress in cancer gene therapy. *Cancer Gene Ther.* 2019;26(11-12):345-346.

11. Kwiatkowska A, Nandhu MS, Behera P, Chiocca EA, Viapiano MS. Strategies in gene therapy for glioblastoma. *Cancers (Basel).* 2013;5(4):1271-1305.

12. Natsume A, Yoshiida J. Gene therapy for high-grade glioma: current approaches and future directions. *Cell Adh Migr.* 2008;2(3):186-191.

13. Seyfried TN, Huysentruyt LC. On the origin of cancer metastasis. *Crit Rev Oncog.* 2013;18(1-2):43-73.

14. Asavarat P, Hajitou A. The phage vaccine against antibiotic resistance. *Lancet Infect Dis.* 2014;14(8):686.

15. Hajitou A, Rangel R, Trepel M, et al. Design and construction of targeted AAVP vectors for mammalian cell transduction. *Nat Protoc.* 2007;2(3):523-531.

16. Hajitou A, Trepel M, Lilley CE, et al. A hybrid vector for ligand-directed tumor targeting and molecular imaging. *Cell.* 2006;125:385-398.

17. Lai TH, Fong YC, Fu WM, Yang RS, Tang CH. Stromal cell-derived factor-1 increase alphavbeta3 integrin expression and invasion in human chondrosarcoma cells. *J Cell Physiol.* 2009;218(2):334-342.

18. Suwan K, Yata T, Waramit S, et al. Next-generation of targeted AAVP vectors for systemic transgene delivery against cancer. *Proc Natl Acad Sci U S A.* 2019;116(37):18571-18577.

19. Stoneham CA, Hollinshead M, Hajitou A. Clathrin-mediated endocytosis and subsequent endo-lysosomal trafficking of adenovirus/phage. *J Biol Chem.* 2012;287(43):35849-35859.

20. Medler J, Wajant H. Tumor necrosis factor receptor-2 (TNFR2): an overview of an emerging drug target. *Expert Opin Ther Targets.* 2019;23(4):295-307.

21. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ.* 2003;10(1):45-65.

22. Josephs SF, Ichim TE, Prince SM, et al. Unleashing endogenous TNF-alpha as a cancer immunotherapeutic. *J Transl Med.* 2018;16(1):242.

23. Paoloni MC, Tandle A, Mazcko C, et al. Launching a novel preclinical infrastructure: comparative oncology trials consortium directed therapeutic targeting of TNFalpha to cancer vasculature. *PLoS ONE.* 2009;4(3):e4972.

24. Aurnhammer C, Haase M, Muether N, et al. Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. *Hum Gene Ther Methods.* 2012;23(1):18-28.

25. Majhen D, Nemet J, Richardson J, et al. Differential role of alphavbeta3 and alphavbeta5 integrins in internalization and transduction efficiencies of wild type and RGD4C fiber-modified adenoviruses. *Virology Res.* 2009;139(1):64-73.

26. Przystal JM, Waramit S, Pranjol MZI, et al. Efficacy of systemic temozolomide-activated phage-targeted gene therapy in human glioblastoma. *EMBO Mol Med.* 2019;11(4):e492.

27. Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants—past, present and future. *Cytokine Growth Factor Rev.* 2014;25(4):453-472.

28. Jiang Y, Yu M, Hu X, et al. STAT1 mediates transmembrane TNF-alpha-induced formation of death-inducing signaling complex and apoptotic signaling via TNFR1. *Cell Death Differ.* 2017;24:660-671.

29. Wajant H, Siegmund D. TNFR1 and TNFR2 in the control of the life and death balance of macrophages. *Front Cell Dev Biol.* 2019;7:91.

30. Kia A, Przystal JM, Nianiaris N, Mazarakis ND, Mintz PJ, Hajitou A. Dual systemic tumor targeting with ligand-directed phage and Grp78 promoter induces tumor regression. *Mol Cancer Ther.* 2012;11(12):2566-2577.

31. Przystal JM, Umukoro E, Stoneham CA, et al. Proteasome inhibition in cancer is associated with enhanced tumor targeting by the adeno-associated virus/phage. *Mol Oncol.* 2013;7(1):55-66.

32. Reardon DA, Nabors LB, Stupp R, Mikkelsen T. Clengiteid: an integrin-targeting arginine-glycine-aspartic acid peptide with promising activity for glioblastoma multiforme. *Expert Opin Investig Drugs.* 2008;17(8):1225-1235.

33. Martinez-Reza I, Diaz L, Garcia-Becerra R. Preclinical and clinical aspects of TNF-alpha and its receptors TNFR1 and TNFR2 in breast cancer. *J Biomed Sci.* 2017;24(1):90.

34. Hajitou A, Lev DC, Hannay JAF, et al. A preclinical model for predicting drug response in soft-tissue sarcoma with targeted AAVP molecular imaging. *Proc Natl Acad Sci U S A.* 2008;105(11):4471-4476.

35. Tandle A, Hanna E, Lorang D, et al. Tumor vasculature-targeted delivery of tumor necrosis factor-alpha. *Cancer.* 2009;115(1):128-139.

36. Yuan Z, Syrkin G, Adem A, et al. Blockade of inhibitors of apoptosis (IAPs) in combination with tumor-targeted delivery of tumor necrosis factor-alpha leads to synergistic antitumor activity. *Cancer Gene Ther.* 2013;20(1):46-56.

37. Smith TL, Yuan Z, Cardo-Vila M, et al. AAVP displaying octreotide for ligand-directed therapeutic transgene delivery in neuroendocrine tumors of the pancreas. *Proc Natl Acad Sci U S A.* 2016;113(9):2466-2471.

38. Dobroff AS, D’Angelo S, Eckhardt BL, et al. Towards a transcriptome-based theranostic platform for unfavorable breast cancer phenotypes. *Proc Natl Acad Sci U S A.* 2016;113(45):12780-12785.

39. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science (New York, NY).* 1998;279(5349):377-380.

40. Hemminki A, Belousova N, Zinn KR, et al. An adenovirus with enhanced infectivity mediates molecular chemotherapy of ovarian cancer cells and allows imaging of gene expression. *Mol Ther.* 2001;4(3):223-231.

41. Krag DN, Shakla GS, Shen G-P, et al. Selection of tumor-binding ligands in cancer patients with phage display libraries. *Cancer Res.* 2006;66(15):7724-7733.