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A strategy for actualization of active targeting nanomedicine practically functioning in a living body

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ABSTRACT

Designing nanocarriers with active targeting has been increasingly emphasized as for an ideal delivery mechanism of anti-cancer therapeutic agents, but the actualization has been constrained by lack of reliable strategy ultimately applicable. Here, we designed and verified a strategy to achieve active targeting nanomedicine that works in a living body, utilizing animal models bearing a patient's tumor tissue and subjected to the same treatments that would be used in the clinic. The concept for this strategy was that a novel peptide probe and its counterpart protein, which responded to a therapy, were identified, and then the inherent ability of the peptide to target the designated tumor protein was used for active targeting *in vivo*. An initial dose of ionizing radiation was locally delivered to the gastric cancer (GC) tumor of a patient-derived xenograft mouse model, and phage-displayed peptide library was intravenously injected. The peptides tightly bound to the tumor were recovered, and the counterpart protein was subsequently identified. Peptide-conjugated liposomal drug showed dramatically improved therapeutic efficacy and possibility of diagnostic imaging with radiation. These results strongly suggested the potential of our strategy to achieve *in vivo* functional active targeting and to be applied clinically for human cancer treatment.

1. Introduction

Drug delivery systems using nanocarriers have great advantages for cancer treatment, such as reduced toxicity, increased tumor accumulation, and effective targeted delivery into tumor lesions [1,2]. Many nanocarriers have been pursued for their passive targeting via the enhanced permeability and retention (EPR) effect. However, passive targeting has weaknesses, including low bioavailability and secondary toxicity caused by low selectivity [3,4]. To overcome the limitations of passive targeting, active targeting has recently been emphasized [5,6]. Active targeting can deliver a high amount of cytotoxic drugs by conjugation of targeted molecules [7]. Active targeting can also lead to a theranostic system. This has recently been spotlighted [8,9] because of its ability to simultaneously facilitate diagnosis and therapy of cancers with prognostic monitoring and minimal toxicity. The most crucial factor that determines the success or failure of active targeting is the practical efficacy of the ligand [10–17]. Peptides are well-suited for active targeting, with chemical and physical advantages that

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improve *in vivo* feasibility [18]. To obtain the optimal peptide that is effective *in vivo* and instantly applicable in the clinical setting, *in vivo* peptide screening in an animal model may be the best approach.

To create an ideal model representing a patient's tumor microenvironment and to verify our strategy, a gastric cancer (GC) patient's tumor tissue was implanted into an immunodeficient mouse to produce a patient-derived xenograft (PDX) model, which was used to discover a marker protein differentially expressed after radiation priming and its corresponding peptide ligand. GC is the second leading cause of cancer-related death worldwide [19]. In the United States, GC-related deaths rose to approximately 11,000 patients in 2014 [20]. The incidence and pathology of GC vary significantly between the East and the West. Since half of the global incidences occur in Eastern Asia, it is considered more seriously in the East [21]. For early-stage GC, surgical resection of the whole tumor leads to a high cure rate and is the mainstay of treatment [22]. Although the 10 year overall survival rate of patients with resected GC is 65%, 3–42% of patients develop metastatic and advanced GC [23–25]. In addition, many GCs are diagnosed at an advanced stage, at which point they are no longer resectable, because GC is commonly asymptomatic at early stages. Other conventional treatments, including radiotherapy and chemotherapy, are not effective for advanced and/or metastatic GC, and the prognosis of those patients remains poor [19,26,27]. Adjuvant radio-chemotherapy is generally recommended after surgery and results in improved survival, but recurrence and metastasis occur at a higher rate locally and in vicinity regions [28,29]. Radiotherapy can be administered, sometimes to improve resectability by downstaging or downsizing tumors [30] and more often for palliation or reduction of symptoms for patients with far-advanced GC. Although the patients who receive radiotherapy are those with very poor prognosis and radiotherapy currently has limitations for patients with advanced GC, exploiting the molecular changes caused by radiation could lead to a breakthrough that would improve the GC cure rate.

In response to radiation, the expression of molecules changes in the tumor tissue, including in cells, on the cell surface, and conjunctional areas [31]. Those molecules could be associated with radiation-mediated anti-cancer functions and may also act as markers of radiotherapeutic efficacy. Meanwhile, ligand molecule-like peptide that targets these radiation-induced marker proteins could also be of great interest. A peptide ligand that actively binds to these differentially expressed marker proteins could effectively function as a guide for precision drug delivery and diagnostic imaging [32]. Therefore, we intended sought to exploit the differentially expressed proteins in radiation-treated tumor tissue and the peptide ligand that specifically targets them for a novel strategy to maximize the efficacy of radiotherapy for advanced GC (Scheme 1. Schematic illustration of the strategy for gastric cancer cure).

Herein, we introduce a novel strategy that can eventually be applied to achieve practical and active targeting nanomedicine based on the results of the patient-representative model used in this study.

2. Materials and methods

2.1. Generation of PDXs

A human GC tissue fragment was implanted once subcutaneously into the flank of a NGS mouse, and the resulting tumor was used to produce a Balb/c nude mouse model bearing human GC on its right hind limb for all experiments. All animal experiments were performed according to the guidelines approved by the Institutional Review Board and Animal Care Committee of Asan Medical Center.

2.2. Radiation treatment

Radiation treatment was initiated when the average tumor volume reached 100–200 mm^3. The treatment group was locally treated with 2 or 10 Gy of IR using a 6 MV photon beam linear accelerator (CL/1800, Varian Medical System Inc., Palo Alto, CA).

2.3. In vivo peptide screening

A phage-display peptide library (Ph.D.-C7C Phage display peptide library; New England Biolabs, Beverly, MA) was used for *in vivo* peptide screening. Biopanning procedures were performed as described in a previous study [33] with some modifications. PDX mice with GC were injected with 1.0×10^9 pfu of the random library via the tail vein 24 h after irradiation. After five rounds of *in vivo* biopanning, phage plaques were randomly picked from soft agar, and their ssDNA was extracted for sequencing analysis. The sequences were analyzed by an automatic sequencing service (Macrogen Inc., Seoul, Korea) using the M13-96 gIII sequencing primer.

2.4. Phage labeling and near-infrared fluorescence imaging

Phages were labeled with Cy5.5 bis *N*-hydroxysuccinimide ester by following the manufacturer's instructions (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After the conjugation, Cy5.5-labeled phages were precipitated by polyethylene glycol, and the Cy5.5-phage conjugation efficiency was determined by a fluorescence measurement system.

NIR images were obtained using an *In Vivo* Imaging System (IVIS) (Perkin Elmer, Waltham, MA). The radiance (photons/s/cm^2/sr) was measured and normalized in the ROI by the provided program.

2.5. Screening for the counterpart to the P1 peptide using a T7 phage human cDNA library

Goat anti-mouse IgG magnetic beads were washed in PBS and blocked in 1% BSA for 1 h. The beads were incubated with a mouse anti-M13 antibody and the P1 peptide phage (1×10^9 pfu) for 2 h at room temperature. The beads were washed three times with PBS, and the T7 bacteriophage human normal cDNA library (1×10^8 pfu) (Novagen, Madison, WI) was added and incubated at 4 °C overnight. T7 phages were eluted with 1% sodium dodecyl sulfate (SDS) and amplified using the *E. coli* host strain BLT5616 for the next round of biopanning. Three rounds of biopanning were performed, and the selected phages were extracted for sequencing analysis.

2.6. Real-time quantitative PCR

The mRNA purification was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The cDNA was generated using the M-MLV cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA) from total RNA isolated from gastric PDX tumor tissues. The final cDNA was mixed with FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Penzberg, Germany) and 10 pmol of each primer. RT-qPCR was performed in a total volume of 12 μl. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The Real-Time PCR System software (Applied Biosystems, Darmstadt, Germany) was used for RT-qPCR data analysis.