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Screening and identification of a specific peptide for targeting hypoxic hepatoma cells

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ABSTRACT

The biological behaviors of residual hepatoma cells after transarterial embolization therapy, which exist in a hypoxic or even anaerobic tumor microenvironment, differ from the tumor cells under normoxic conditions. This study aimed to use a phage display peptide library for *in vivo* and *in vitro* screening to obtain a peptide which could specifically bind to hypoxic hepatoma cells, allowing further targeted diagnosis and treatment for liver cancer. In this study, hypoxic hepatoma cells HepG2 (targeted cells), and normal liver cells HL-7702 (control cells), were utilized to perform three rounds of *in vitro* screening using a phage-displayed 7-mer peptide library. In addition, hypoxic HepG2 were subcutaneously injected into nude mice to establish a hepatocarcinoma model, followed by performing three rounds of *in vivo* screening on the phages identified from the *in vitro* screening. The products from the screening were further identified using ELISA and immunofluorescence staining on cells and tissues. The results indicated that the P11 positive clone had the highest binding effect with hypoxic hepatoma cells. The sequence of the exogenous insert fragment of P11 positive clone was obtained by sequencing: GSTSFSK. The binding assay indicated that GSTSFSK could specifically bind to hypoxic hepatoma cells and hepatocarcinoma tissues. This 7-mer peptide has the potential to be developed as an useful molecular to the targeting diagnosis and treatment of residual hepatoma cells after transarterial chemoembolization.

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1. Introduction

Transarterial embolization (TACE) has been a major therapeutic approach for the management of advanced hepatocellular carcinoma (HCC). TACE in HCC treatment relies on slowing down or blocking blood supply to tumors, which results in ischemic necrosis in cancerous tissue [1,2]. However, although most tumor cells die after the therapeutic embolization, a few tumor cells may survive. It is important to note that these residual tumor cells, which survive under hypoxic even anaerobic microenvironment, ultimately becomes the source of cancer recurrence and metastasis [3,4]. With this in mind, both the detection and targeted therapy of these residual tumor cells are essential in effectively treating HCC.

Previous studies have suggested that hepatoma cells that underwent TACE had higher expression of hypoxia-inducible transcription factor-1α (HIF-1) than hepatoma cells that did not undergo TACE [3,5]. HIF-1α binds to promoter elements of downstream target genes and upregulates their expression, leading to changes in biological behaviors of tumor cells – including tumor metabolism, angiogenesis, cell proliferation and differentiation, drug resistance, and endocytosis of anticancer drugs [6–10]. Cifani et al. [11]. recently reported the effects of hypoxia on the membrane proteome of tumor cells, which demonstrated that chronic hypoxia affected the expression of membrane protein in tumor cells. Some membrane proteins that are specifically expressed under hypoxic conditions might be involved in resistance to anticancer therapies and could be potential therapeutic targets. Hsu et al. [12]. reported that hypoxia altered the activities of the tyrosine kinase receptor family, resulting in changes in Clathrin-mediated endocytosis. These studies suggest that residual hepatoma cells that remain after TACE could be able to survive in hypoxic or anaerobic microenvironments. In these microenvironments, the phenotypes of the tumor cells, including their drug binding sites and endocytosis

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pathway, could possibly differ from normoxic tumor cells. Little is known about these specific changes, but phage display peptide library technology could be used for screening the peptide that bind to hypoxic hematoma cells specifically [13].

In this study, normal liver cells (HL-7702) were used as the control cells and hypoxic hepatoma cells (HepG2) were used as targeted cells for three rounds of *in vitro* screening. In order to establish an HCC animal model, hypoxic hepatoma cells were subcutaneously injected into nude mice and the *in vitro* screened products were utilized in three rounds of *in vivo* screening in the HCC mouse model. The peptide GSTSFSK, obtained through the *in vitro* and *in vivo* screening, had significantly higher binding specificity on hypoxic HepG2 than normoxic HepG2 and HL-7702. This peptide could also bind to tumor tissue isolated from the nude mice HCC model. This study suggested that GSTSFSK might play an important role in the targeted diagnosis and treatment of residual hepatoma cells after TACE.

2. Materials and methods

2.1. Materials

Hepatoma cells, HepG2, used in this study were stored and passaged in standard laboratory conditions in our laboratory. The normal liver cells HL-7702 were purchased from the China Center for Type Culture Collection (Wuhan University, Hubei, China). Nude mice were purchased from the Laboratory Animal Center, Peking University, Beijing, China. The Ph.D.™-7 Phage Display Peptide Library Kit was purchased from New England BioLabs Inc. (Ipswich, MA). Rabbit anti-M13 phage antibodies were was purchased from Abcam (Cambridge, UK). Fluorescein (FITC)-labeled sheep anti-rabbit immunoglobulin (IgG) antibodies were purchased from Sigma-Aldrich (St. Louis, USA).

Bovine serum albumin (BSA) and RPMI 1640 medium were purchased from Invitrogen (Carlsbad, CA). The phage single-strand DNA extraction kit was purchased from Shanghai Kang Lang Biological Technology Co., Ltd. (Shanghai, China). Phage single-strand DNA was sequenced by Sango Biotech, Co., Ltd. (Shanghai, China).

Nude mice were housed under specific pathogen-free conditions in accordance with the guideline of the Experimental Animal Center, Huazhong University of Science and Technology, Hubei, China. In order to establish the HCC mouse model, 200 μL of 1×10^{11} hypoxic HepG2 cells (diluted in RPMI-1640 medium) was subcutaneously injected into the lateroventral region of 4-week old nude mice. After 4–6 weeks, tumors that were ~1 cm^3 size were used for *in vivo* screening and binding assays.

2.2. In vitro and in vivo panning

Hypoxic (94% N_2, 5% CO_2, 1% O_2, same as below) hepatoma cells HepG2 (targeted cells) and normoxic liver cells HL-7702 (adhesive cells) underwent three rounds of *in vitro* subtractive screening, as described in the manufacturer's instructions for the Ph.D.™-7 Phage Display Peptide Library Kit. The same input amount of phage was used in each round of screening.

The procedure for *in vitro* subtractive screening was performed as follows: Hypoxic HepG2 and normal human liver cells HL-7702 at logarithmic growth phase were digested with 0.25% trypsin (containing 0.02% EDTA) and seeded on a PLL (poly-L-lysine) precoated 6-well plate to culture until cells were adhesive. After discarding the culture medium, RPMI 1640 medium (1% BSA) was used to block the cells for ~1 h. After washing thrice with PBS, a phage display library (2×10^{11} pfu) was incubated with HepG2 at 37 °C for 1 h and shaking at 100 rpm. After discarding the unbound phage, the cells were washed in TBST-01 five times, followed by adding 100 μL of 0.2 M glycine-HCl (pH 2.2) solution (1 mg/mL FCS) per well to incubate for 10 min and shaking at 100 rpm. The solution was then transferred into a centrifuge tube containing 15 μL of 1 M Tris-HCl (pH 9.1) neutralization solution, followed by adding the neutralization solution to the blocked normal human liver cells HL-7702 and incubating at 37 °C for 1 h. The unbound phage were collected and amplified, purified, and tittered according to the manufacturer's instructions.

For *in vivo* panning, the phage display library (1×10^{11} pfu) was injected through the tail veins of the mouse. After 15 min, mice were sacrificed and perfused through heart with 100 mL of D-Hank's buffer. Tumor tissues were then collected, minced, and suspended in 2 mL D-Hank's buffer for phage tittering. For phages amplification, the remaining tumor tissue was minced and mixed with 20 mL of *E.coli* ER2238 culture (at early-log stage) and incubated at 37 °C with vigorous shaking for 4.5 h, followed by purified and tittered for the next round of panning. After the three round of *in vivo* panning, 60 phage clones were randomly picked out from tittered phage plaques for the following cellular ELISA.

2.3. Phage binding assay by cellular ELISA

HepG2 and HL-7702 liver cells were added into 96-well plate (1×10^4 cells/well) and cultured for 24 h (HepG2 were cultured under hypoxic condition as described previously), followed by 1 h of serum-free treatment and a subsequent washing step. Cells were fixed in 4% paraformaldehyde for 20 min and then washed in PBS. Both cells were washed thrice in PBST-05 (PBS with 0.05% Tween 20), and blocked in 2% PBS-BSA solution for 1 h. Each selected phage clone was added to the cells at 10^{10} pfu per well, and the cells were incubated at 37 °C for 1 h. After washing thrice with PBST-05 (1 min each), 100 μL of 1:5000 rabbit anti-M13 antibodies diluted with blocking buffer was added into each well and incubated at 37 °C for 1 h. After discarding the primary antibody and washing with PBST-05, 100 μL of 1:2000 HRP-sheep anti-rabbit immunoglobulin diluted with blocking buffer was added to each well and incubated at 37 °C for 1 h. The cells were washed thrice with PBST-05 (1 min each), and then incubated with 3, 3′, 5, 5'-tetramethylbenzidine (TMB) (Sigma, Saint Louis, USA) at 37 °C for 30 min. An HCl solution was added to terminate the color development, followed by reading the optical density (OD) of each sample at 450 nm in a microplate reader (Thermo Scientific, MK3, USA). Irrelevant phage clone (IRP, an amplified phage randomly selected from the original pahge peptide library) was selected as a control. Selectivity is calculated by the following formula: Selectivity $= (OD_{S1} - OD_{C1})/(OD_{S2} - OD_{C2})$. Selectivity that was>2 was identified as positive phage in this study.

OD_{S1} and OD_{C1} represented ODs of the selected phage and the control phage in hypoxic HepG2, respectively; while OD_{S2} and OD_{C2} represented the ODs of the selected phage and the control phage in HL-7702, respectively.

2.4. Cell immunofluorescence and image analysis

Normoxic and hypoxic HepG2, and HL-7702 cells, were digested with 0.25% trypsin, suspended in RPMI to 10^4 cells/mL, and then grown on a 24-well chamber slide overnight. Cells were washed thrice with PBS for and subsequently fixed with 4% paraformaldehyde at room temperature for 30 min, followed by washing with PBS and blocking in 1% PBS-BSA at room temperature for 30 min to prevent non-specific binding. The selected positive hages (1×10^{10} pfu) diluted in PBS was added to the cells respectively, while the same volume of PBS and IRP phages were used as negative control, and all cells were incubated at room temperature for 1 h. After washing the cells three times with PBST-01 (1 min

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