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Identification of stem-like cells in non-small cell lung cancer cells with specific peptides

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ABSTRACT

Recent studies indicate that tumor maintenance, metastasis and drug-resistance are mainly conducted by a small subset of cancer cells which are termed cancer stem cells (CSCs) or cancer stem-like cells (CSLCs). Successful identification of CSCs/CSLCs might lead to discovery of the novel and effective therapeutic targets for cancers. In our study, **lung CSCs/CSLCs were enriched by sphere-forming assay**. **Screening and selection of specific binding peptides for lung CSCs/CSLCs were performed with bacterial surface display method**. Selected peptide named HCBP-1 exhibited highest specific binding capability as examined by flow cytometry and fluorescence microscopy. Drug-resistant lung CSCs/CSLCs might be characterized with HCBP-1 peptide and several microRNAs related to the stem-like properties were discriminatively expressed in HCBP-1$^+$ subpopulation. Moreover, at least two distinct subpopulations in H460 tumor sphere cells could be distinguished by HCBP-1 peptide. Thus, **a new method was established to identify lung CSCs/CSLCs, which provided robust approaches for the research of CSCs/CSLCs**.

Introduction

Cancer is a leading cause of death in the world. Although the lives of cancer patients have been extended by continuously improved therapies, the overall survival rate remains particularly low. To devise more effective strategies, it is critical to identify which population of cancer cells is responsible for tumor progression [1–3]. Recent studies indicate that only a small subset of cancer cells termed cancer stem cells (CSCs) or cancer stem-like cells (CSLCs) would contribute to tumor maintenance, metastasis and drug-resistance [1,4].

Identification of CSCs/CSLCs might accelerate the understanding of tumor biology and give a clue to develop more effective treatments by targeting tumorigenic cells. Currently, identification of CSCs/CSLCs is highly dependent on surface markers that shared with normal stem cells [2]. For example, CD133, a reliable surface marker of CSCs/CSLCs found in many types of tumors [5–7], was initially described as a surface marker for human hematopoietic progenitor cells [8]. Nevertheless, recent studies indicate that CD133 does not distinguish CSCs/CSLCs from non-CSCs/CSLCs in some cases [9–11]. There are accumulating evidences that the heterogeneity of CSCs/CSLCs was not only observed among different types of tumors but also among the subtypes of a tumor [2,4], which leads to the difficulty to obtain the common CSCs/CSLCs markers for a certain tumor.

Progress in CSCs/CSLCs research has been hampered by the lack of surface markers for some tumors [4]. Alternatively, CSCs/CSLCs have been enriched by functional assays which also shared with tissue stem cells, such as sphere-forming assay [5], Hoechst 33342 dye efflux assay [12] and Aldefluor assay [13]. Currently, serum-free sphere cultures represent the most recognized method to enrich and expand CSCs/CSLCs *in vitro* [2,5]. However, CSCs/CSLCs subpopulation defined by sphere-forming assays has been reported to overlap poorly with subpopulations defined by other functional assays, which reflected that CSCs/CSLCs might be composed by multiple subpopulations [14,15]. Since tumor spheres are heterogeneous cell clusters [5,16], the phenotypic profiles of CSCs/CSLCs in tumor spheres still remain unclear. Nevertheless, serum-free sphere culture could be used to obtain sufficient CSCs/CSLCs *in vitro*, which is essential for developing targeting molecules for recognizing CSCs/CSLCs.

Targeting molecules such as peptides have been used in a variety of fields, ranging from cell imaging to targeting therapies [17]. Under circumstances that definitive information of the target cell

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surface antigens is unknown, specific binding peptides have been successfully sorted out using bacterial surface display method [17,18]. In this study, lung CSCs/CSLCs would be enriched using sphere-forming assay. Specific binding peptides for lung CSCs/CSLCs would be obtained by bacterial surface display method. Furthermore, we intended to develop a new method to distinguish lung CSCs/CSLCs among different subpopulations based on the specific binding peptides.

Materials and methods

Cell culture

Human lung cancer cell lines (A549 cells, H1299 cells and H460 cells) were maintained in RPMI-1640 medium (Hyclone). Human embryonic lung fibroblast cell line (HLF) was cultured with high glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone). Human mesenchymal stem cells (MSCs) were maintained in DMEM-F12 (Hyclone). All media were supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Hyclone). Cells were cultured in a 5% CO_2 humidified incubator at 37 °C. Human lung cancer cell lines and HLF cells were kind gifts from Zhang [19–21] (Guangzhou institutes of biomedicine and health, CAS, China). MSCs were kindly provided by Yang [22] (Suzhou Institute of Nano-Tech and Nano-Bionics, CAS, China).

To obtain tumor spheres, single tumor cells from three lung cancer cell lines were separately plated at clonal density (1×10^4 cells/ml) in the serum-free medium DMEM-F12, supplemented with 20 ng/ml basic fibroblast growth factor (bFGF, PeproTech), 20 ng/ml epidermal growth factor (EGF, PeproTech) and 2% B27 (Gibco). For serial passages, tumor spheres were dissociated after 2 weeks of culture by trypsinization with 0.25% Trypsin and 0.02% EDTA for 2 min at 37 °C and physical separation by pipetting up and down several times, followed by re-plating the single cells in fresh medium.

Self-renewal and proliferation capabilities examination

H460 tumor sphere cells were dissociated and diluted to a density of 500–1000 cells/ml using limiting dilution assay. Then, a 2-μl aliquot was added to each well of 96-well plates containing 50 μl serum-free medium supplemented with growth factors. Wells containing only one viable cell were marked and observed everyday. Each well was supplemented with 50 μl growth medium every three days.

Parental cells and sphere cells were dissociated and plated into 24-well plates at 1.2×10^4 cells/ml in serum-free medium without growth factors. Quantification of viable cells was performed by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide) assay after 1 week of culture.

Bacterial surface display library screening, selection and analysis

Bacterial surface display library was a kind gift from Daugherty [17] (University of California, Santa Barbara, United States). The random library of $X_2CX_7CX_2$ (13mer peptides) was comprised of 10^9 members. The 13mer peptides were displayed on the surface of bacteria by fusion to the extracellular N-terminus of a circularly permuted variant of outer membrane protein OmpX (CPX) of *Escherichia coli*. These bacteria could express green fluorescent protein (GFP) and CPX simultaneously under the control of the *araBAD* promoter.

Isolation of peptide-bacteria that bind to target cells was performed as previously described [17,18] with some modification. Briefly, the entire screening progress includes four rounds of co-sedimentation and four rounds of fluorescence-activated cell sorting (FACS). Before screening by FACS, bacteria were selected using co-sedimentation to reduce the library size. Then, the enriched bacterial libraries were sorted with a FACS Aria II (Becton Dickinson, San Jose, CA). Bacteria from last round of selection were plated on LB-agar plates and thirty monoclonal bacteria were picked. Binding capability and specificity of individual clones were determined by flow cytometric analysis and peptide sequences were determined via DNA sequencing performed by GENEWIZ Inc. (Suzhou, China).

Binding analysis of soluble peptides

Two cyclic peptides with disulfide bond formation (HCBP-1: GGLGCFPEGEMACWWSGGSGK and control: GSSGCSSGSSGSCSGSSGSSK) were synthesized and labeled with fluorescein isothiocyanate (FITC) at the N-terminus by Shanghai Bootech bioscience and technology Co., Ltd. (Shanghai, China).

For flow cytometric analysis, tumor spheres and parental cells were dissociated into single cells and resuspended (5×10^5 cells/ml) in phosphate buffered saline (PBS, PH 7.4). Cells were incubated with FITC labeled peptides at a final concentration of 0.5 μM for 30 min on an inversion shaker at 4 °C and samples were analyzed by flow cytometry.

For fluorescence microscope analysis, tumor spheres and parental cells on coverslips were fixed with 4% formaldehyde for 10 min at room temperature. After blocked with 3% bovine serum albumin (BSA, Roche) for 10 min, tumor spheres and parental cells were incubated with 3.3 μM FITC labeled peptides for 30 min at room temperature. Cell nuclei were counterstained with Hoechst 33342 (2 μg/ml, Sigma–Aldrich Co.) for 10 min. The slides were visualized and photographed with fluorescence microscopy (Nikon Ti, Nikon Corp. Japan).

Determination of peptide K_D value

The apparent dissociation constant (K_D) of HCBP-1 was determined using a flow cytometry-based assay [23]. Sphere cells (5×10^5 cells/ml) were incubated separately with FITC-HCBP-1 peptide in gradient dilutions from 0.025×10^{-6} to 0.833×10^{-6} M at 4 °C until equilibrium was reached. Relative fluorescence intensity of samples were detected and analyzed by flow cytometry. The inverse of the fluorescence intensity was plotted as a function of the inverse of FITC-HCBP-1 peptide concentration to determine K_D by the Lineweaver–Burk method.

Examination of drug resistance

Parental cells were treated with cisplatin (with a final concentration of 20 μM) for 1 day, 3 days, 5 days and 7 days respectively. Cells were dissociated and incubated with FITC labeled peptides with a final concentration of 0.5 μM for 30 min at 4 °C. Samples were analyzed by flow cytometry.

Extraction of microRNA and microarray screening

After H460 tumor sphere cells labeled with FITC-HCBP-1 peptide, HCBP-1^+ and HCBP-1^- sphere cells were sorted by FACS. Total RNA including microRNAs (miRNAs) from H460 parental cells, sphere cells, HCBP-1^+ and HCBP-1^- sphere cells were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

The miRNA microarray assay was performed as previously described using a fluorescent label-free strategy [24]. Total RNA (2.0 μg) from each sample were hybridized onto the miRNA microarray, which included 2018 mature human miRNA sequences (Sanger miRBase, release 19.0). Differently expressing miRNAs (fold change $\geqslant 1.3$) were selected for further analysis.

miRNA expression analysis using qRT-PCR

The microarray data was validated by qRT-PCR. Total RNA (1.0 μg) from each sample were used to generate cDNA by using PrimeScript™ RT reagent Kit (TaKaRa, RR037A) with special stem-loop primer for miRNA. Human small nuclear U6 RNA was used as the internal reference for normalization. The primers for qRT-PCR were designed as previously described [25]. Real-time qPCR was performed on a Roche LightCycler System (Roche Diagnostics) using SYBR® Premix Ex Taq™ II (TaKaRa, RR820A). Melting curves were generated for each qRT-PCR reaction to verify the specificity. All the reactions were performed in triplicate and relative fold changes were calculated by the equation $2^{-\triangle\triangle Ct}$. The sequences of the primers used in qRT-PCR were listed in Supplementary Table S1.

Statistically analysis

All these experiments were independently replicated three or more times and the data were presented as mean ± standard deviation (S.D.). Comparisons to untreated controls were carried out using Student's *t*-test (Microsoft Excel, Microsoft Corporation, USA). *P*-value < 0.05 was considered to be statistically significant.

Results

Tumor spheres were generated from NSCLC cell lines with the ability of self-renewal and proliferation

To explore whether lung CSCs/CSLCs could be enriched by sphere-forming assay, A549, H1299 and H460 cells were cultured in serum-free medium. We observed that most cancer cells died and only a small subset of cells formed tumor spheres in the first couple of weeks. Tumor spheres were generated from all three cell lines (Fig. 1A and Supplementary Fig. S1) but at different efficiencies. H460 cells displayed the highest sphere-forming efficiency and A549 displayed the lowest. H1299 and H460 tumor spheres could grow indefinitely in a long-term culture (more than 20 passages), but A549 tumor spheres expanded only six passages. H460 tumor spheres between 8 and 20 passages were used for further analyses.