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Effects of lentivirus-mediated silencing of Periostin on tumor microenvironment and bone metastasis via the integrin-signaling pathway in lung cancer

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ABSTRACT

The study aims to investigate the effects of Periostin gene silencing on tumor microenvironment and bone metastasis via the integrin-signaling pathway in lung cancer (LC). LC patients were divided into bone metastasis and non-bone metastasis groups; Healthy volunteers were selected as normal group. ELISA was performed to detect serum Periostin levels and plasma calcium ion concentration. SBC-5 cells were assigned into blank group (without transfection), negative control (NC) group (transfected with empty plasmid), si-Periostin group (transfected with si-Periostin plasmid), si-Integrin-αvβ3 group (transfected with Integrin-αvβ3 siRNA plasmid) and si-Periostin + si-Integrin-αvβ3 group (transfected with si-Periostin and si-Integrin-αvβ3 plasmid). qRT-PCR and Western blotting were performed to determine mRNA and protein expression of Periostin, metastasis-associated factors of tumor microenvironment and integrin signaling pathway-related proteins. CCK-8, scratch test and transwell assay were applied to detect cell proliferation, migration and invasion respectively. Nude mouse models of LC bone metastasis were established. TRAP Staining was employed to measure the number of osteoclasts. Bone metastasis group exhibited higher levels of Periostin compared to normal and non-bone metastasis groups. Si-Periostin, si-Integrin-αvβ3 and si-Periostin + si-Integrin-αvβ3 groups showed decreased Periostin expression, proliferation rate, migration distance, invasive cells, and expressions of metastasis-associated factors of tumor microenvironment and integrin signaling pathway-related proteins compared to blank and NC groups. Similarly, number of osteoclasts and expression of integrin signaling pathway-related proteins were decreased, and bone injury and calcium ion concentration were reduced. The study demonstrated that down-regulation of Periostin expression modulated tumor microenvironment and inhibited bone metastasis by blocking integrin-signaling pathway in LC.

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1. Introduction

In the past few years, the field of lung cancer (LC) research has advanced remarkably, however LC remains to be the leading cause of cancer-related deaths all over the world [1]. Unfortunately, the majority of LC cases are diagnosed at an advanced stage once the cancer has metastasized, thus limiting the possibility of finding a cure [2]. Brain metastasis, for patients suffering from LC, is a widespread secondary location, which is present in approximately 30–40% of LC patients in the trajectory of the disease [3]. The big challenge of early diagnosis of LC remains to be a difficulty leading to a poor prognosis with short survival time [4]. Periostin, an extracellular matrix protein serves as a key factor in both cell motility and adhesion across the tumor microenvironment [5]. It regulates development of several types of human cancers by means of interactions with Rb/E2F1/p14ARF/Mdm2 pathways or PI3K/Akt/survivin pathways, and is related to tumor growth, invasion, as well as metastasis [6–8].

Lately, Periostin over-expression has been shown to result in poor prognosis as well as an increased risk of metastasis in many malignancies, such as lung cancer, gastric cancer, colon cancer, breast cancer, and head and neck cancer [6,9–12]. Periostin is abnormally up-regulated in LC and furthermore shows close associations with epithelial-mesenchyme transition, metastasis, invasion and angiogenesis [13]. High expression of Periostin plays an important physiological role during

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cell growth at dental, bone, and cardiac tissues levels [14]. Periostin can act as a critical regulator of cell survival, angiogenesis and hypoxia-induced cell apoptosis through activation of intracellular singling pathways and binding to the integrins αvβ3, αvβ5 and α6β4, [15]. Sun et al. found that integrins affect cell growth in LC, functioning as an important factor in the development of more efficient cancer treatment strategies [16]. Interestingly, tumor cells interplay with their environment with the help of several transmembrane proteins, including integrins and some intercellular adhesion molecules that not only mediate tumor metastasis and progression, but also support cell-cell interactions [17]. Moreover, Periostin promotes cell adhesion and motility by activation of integrin-mediated signaling, and also enhances osteoblast-osteocyte differentiation [18]. Periostin acting as a tumor-invasive indicator enhances invasion in esophageal cancer tumor microenvironment [19]. However, the role of Periostin in the tumor microenvironment and bone metastasis remains unknown. Thus, we foremost propose a hypothesis that shows down-regulation of Periostin may alter tumor microenvironment and affects development and progression of bone metastasis in LC through its role in the integrin-signaling pathway. In the present study, we also showed the effects of down-regulation of Periostin on proliferation, migration, invasion of human small cell lung cancer (SBC-5) cells with high bone-metastatic potential in vitro and in vivo.

2. Materials and methods

2.1. Ethics statement

The study was approved by The Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and written informed consents were obtained from all subjects.

2.2. Study subjects

The study population consists of a total of 213 LC patients from the Department of Oncology in Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology from January 2014 and January 2016. All patients were assigned into a bone metastasis group ($n = 101$) and non-bone metastasis group ($n = 112$). Inclusion criteria was as follows: (1) complete data were obtained from patients who were pathologically diagnosed as suffering from LC [20]; (2) LC patients undergone bone metastasis were confirmed by bone scans, X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). The calculated mean age of patients in the non-bone metastasis group was 56.43 ± 8.75 years (range 37–73 years), including 65 males and 47 females. The aforementioned patients were established devoid of any tumor tissues in other parts of body. The calculated mean age of patients in the bone metastasis group was 57.50 ± 8.65 years (range 35–73 years), including 59 males and 42 females. Additionally, a total of 73 healthy volunteers aged 23 to 69 years (52 males and 21 females, calculated mean age: 50.37 ± 9.34 years) undergoing medical examination at the same period were selected as the normal group.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Empty stomach venous blood samples (5 ml) were obtained from each subject. 30 min prior to extraction, the blood was centrifuged at rate of 3000 r/min for 15 min to separate the serum from the blood which was subsequently refrigerated at −80 °C. Serum Periostin was employed with ELISA Kit (No. PG48T; Shanghai Yan Ji Bio Technology Co., Ltd. Shanghai, China). Serum samples were diluted at proper proportions, and standard (50 μl) and diluted samples (50 μl) were added into the ELISA plate covered with membrane The contents of the ELISA plate were discarded after the reaction, and 50 μl of ELISA reagent was added into each well for incubation at 37 °C for 30 min. 50 μl of Chromogenic agent A and 50 μl of chromogenic agent B were mixed together in each well after being washed with phosphate buffered saline (PBS) three times, and at that juncture chromogenic reaction was carried out at 37 °C in the dark for 15 min, followed by the addition of 50 μl stop buffer to halt the reaction. Optical density (OD) value was measured at 450 nm wavelength in a microplate reader. Calculation of the final density of the samples was carried out using the corresponding concentration on the microplate reader, which was multiplied by dilution.

2.4. Construction of Periostin siRNA lentiviral vector

Periostin mRNA (Genbank: NM_002473.4) was employed as the template strand, and the sequences of Periostin siRNA were as follows: sense strand, 5′-GCAACGUGAAUGUUGAAUUTT-3′; antisense strand, 5′-AAUUCAACAUUCACGUUGCTC-3′. Human Integrin-αv and Integrin-β3 mRNA sequences were employed as the template strand, and the sequence of Integrin-αv siRNA was as follows: 5′-GUAGUCA AUUCUAUCAGAdTdTdTdTCAUCAGUUAGAGAUAGUCU-3′, and the sequence of Integrin-β3 siRNA was as follows: 5′-GUGCAAUCUUGU ACGUAAAdTdTdTdTCACGUUAGAACAUGCAUUU-3′. Simultaneously, empty plasmids were selected as the negative control group, and lentivirus vectors were constructed by the Shanghai Genechem Co. Ltd. (Shanghai, China). Single-stranded DNA oligosaccharide fragments were synthesized, and subsequently double-stranded DNA of oligosaccharide was obtained post-primer annealing. Vector linearization was achieved after utilizing restriction enzyme *Hpa*I and *Xho*I, and followed by lentiviral vector carrying Periostin siRNA sequences construction by connecting to Periostin siRNA sequences through DNA. The recombinant vector was transferred into competent DH5a cells, and positive DH5a cells among the cell culture underwent selection and cloning. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed in order to check for lentiviral vector establishment. Positive clones were amplified post-sequencing, and plasmid isolation kit (No. D0018, Beyotime Institute of Biotechnology, Beijing, China) was used for Periostin siRNA plasmid (si-Periostin) extraction.

2.5. Cell culture and transfection

Human SBC-5 cell line with high bone-metastatic potential was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 medium (CAS: 16000-044; Gibco, Grand Island, NY, USA) was used in order to culture SBC-5 cells supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37 °C. Upon reaching approximately 80–90% confluence, SBC-5 cells were sub-cultured every 2–3 days. The cells meeting the criterion of being in the logarithmic phase of growth were seeded into 6-well plates (3×10^5 cells/well). SBC-5 cells were divided into the following groups: negative control (NC) group (cells transfected with empty plasmids), si-Periostin group (cells transfected with si-Periostin plasmids), si-Integrin-αvβ3 group (cells transfected with si-Integrin-αvβ3 plasmids), si-Periostin + si-Integrin-αvβ3 group (cells transfected with si-Periostin and si-Integrin-αvβ3 plasmids) and blank group (cells without transfection). Cell transfection was achieved using Lipofectamine 3000 (CAS: L3000-015, Invitrogen, California, USA) following the manufacturer's instruction. A day prior to transfection, cells were sub-cultured and seeded into 6-well plates. Upon reaching approximately 90–95% confluence, diluted plasmids were mixed with diluted Lipofectamine 3000 in a serum-free medium. After resting at room temperature for 20 min, the mixture was seeded into each well, and further incubated for 4 h at 37 °C in a 5% CO_2 incubator, and consequently the medium was changed. Further experiments were carried out 48 h after cell transfection.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA Isolation from lung tissues of all subjects and SBC-5 after transfection was carried out using RNA extraction kit (CAS: 74104; Qiagen,

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