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Metastatic potential of lung squamous cell carcinoma associated with HSPC300 through its interaction with WAVE2

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ABSTRACT

The small protein, HSPC300 (haematopoietic stem/progenitor cell protein 300), is associated with reorganization of actin filaments and cell movement, but its activity has not been reported in human cancer cells. Here, we investigated the association of HSPC300 expression with clinical features of lung squamous cell carcinoma. High levels of HSPC300 protein were detected in 84.1% of tumour samples, and in 30.8% of adjacent morphologically normal tissues. The number of primary tumours with elevated HSPC300 levels was significantly higher in primary tumours with lymph node metastases as opposed to those without, and also in tumours from patients with more advanced disease. HSPC300 modulates the morphology and motility of cells, as siRNA knockdown caused the reorganization of actin filaments, decreased the formation of pseudopodia, and inhibited the migration of a lung cancer cell line. We further showed that HSPC300 interacted with the WAVE2 protein, and HSPC300 silencing resulted in the degradation of WAVE2 *in vitro*. HSPC300 and WAVE2 were co-expressed in approximately 85.7% of primary tumours with lymph node metastases. We hypothesize that HSPC300 is associated with metastatic potential of lung squamous cell carcinoma through its interaction with WAVE2.

1. Introduction

Approximately 70% of non-small cell lung cancer (NSCLC) patients are estimated to have clinically detectable or occult metastasis at initial diagnosis, causing 90% of cancer deaths [1]. A better understanding of the metastatic mechanism and the genes involved could potentially thus lead to improved diagnosis and treatment of the disease.

An early and essential step in metastasis is cell migration from the primary tumour into the lymph or blood circulation. Cell movement and adhesion are mediated by dynamic polymerization of actin filaments (F-actin) [2]. A number of molecules have been implicated in this process, including actin-related protein 2/3 (Arp2/3) complex, Cdc42, EGFR, Rac, myosin and suppressors of cAMP receptor/Wiskott-Aldrich syndrome protein family (WASP) verprolin homologue (Scar/WAVE) [3]. The WAVE proteins, consisting of WAVE1, WAVE2 and WAVE3, are composed of multiple conserved domains that include an N-terminal WASP homology domain, a region of basic amino acids, a proline-rich domain, and a C-terminal verprolin/cofilin/acidic domain [4,5]. WAVEs bind both monomeric actin and the Arp2/3 complex via their verprolin/cofilin/acidic domain, and function as a platform to mediate Rac1-induced actin polymerization [5,6]. WAVE2 is essential for lamellipodial formation in fibroblasts [7,8]. Overexpression of WAVE2 protein has been associated with poor clinical outcome in patients with hepatocellular and breast carcinoma tumour [9,10].

It has been reported that all three WAVE isoforms exist in a pentameric heterocomplex with Abelson-interacting protein (Abi), haematopoietic stem progenitor cell 300 (HSPC300, homologue of BRICK1), Nck-associated protein 1 (Nap1, also known as p125NAP1), and Ras-associated protein 1(Sra-1) [11,12]. Among these, HSPC300 is a small protein of 75 amino acids with a presumed coiled-

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coil region that mediates binding to WAVEs [13]; and *Arabidopsis* BRICK1/HSPC300 is critical for the stability of the Arp2/3 activator, WAVE2 [14,15]. However, a role for HSPC300 in human cancers is poorly defined and understood.

Our previous data, however, demonstrated that HSPC300 was one of the genes up-regulated in a differential expression cDNA library derived from NSCLC [16,17]. Here, we have investigated the expression of HSPC300 protein in the tumour tissues of human lung squamous cell carcinoma and its biological effects on the migration of lung cancer cells, and studied the possible underlying molecular mechanism of our findings.

2. Material and methods

2.1. Patients and specimens

Formalin-fixed, paraffin-embedded tissue samples derived from 128 lung squamous cell carcinoma patients (112 males and 16 females) were obtained in the form of tissue microarrays (TMAs) that were constructed in the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences. A total of 158 specimens consisting of 145 human lung squamous carcinoma tissues and 13 adjacent morphologically normal tissues were simultaneously taken from the same group of lung squamous cell carcinoma patients. The tumour samples included 32 primary tumours without lymph node invasion, 75 with lymph node metastasis, and 38 lymph node metastatic tumours (including 24 paired with their corresponding primary tumours). The histological diagnosis of all the tissue samples was re-confirmed by experienced pathologists. The patients were clinically staged according to the international tumour-node-metastasis system; none of them had received chemo- and/or radio-therapy before surgery.

2.2. Lung cancer cell lines

NSCLC cell lines involved in this study were A549, H520, H1299, H2170 (American Type Culture Collection, Manassas, VA, USA), and PG. Among them, PG originated from a human pulmonary giant cell carcinoma exhibited relatively high invasive and metastatic potential [18,19]. Generally, the cells were cultured in RPMI 1640 medium containing 10% foetal calf serum at 37 °C in a 5% CO_2 atmosphere.

2.3. Expression of HSPC300 and/or WAVE2

2.3.1. Immunohistochemistry (IHC) analysis

Slides (5 μm-TMAs) were incubated with a rabbit polyclonal antibody against HSPC300 (Eurogentec, Liege, Belgium) or a rabbit polyclonal antibody against WAVE2 (Santa Cruz, Santa Cruz, CA, USA), respectively, overnight at 4 °C; a biotinylated mouse anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA, USA) was applied for 30 min at 37 °C, followed by a streptavidin-peroxidase antibody (Invitrogen, USA) incubation, followed by nuclei counterstaining with hematoxylin. The IHC stained slides were examined by two pathologists independently and scored as previously described [18]. When investigating the co-expression of HSPC300 and WAVE2, two TMAs-sections from the same tissue samples, both stained with antibodies against HSPC300 and WAVE2, were deemed positive for co-expression.

2.3.2. Immunoblot analysis

Expression of HSPC300 in cell lines was examined by Western blotting probed with a rabbit polyclonal antibody against HSPC300, using β-actin as loading control.

2.4. 'Knocking down' expression of HSPC300 or WAVE2 in lung cancer cells

Double-stranded siRNA oligonucleotides targeting HSPC300 (No. 1: 5′-CGA UAU GUC UUG UCG UUC AdTdT-3′; No. 2: 5′-AAC GGA GAA UAG AGU ACA UdTdT-3′; No. 3: 5′-UUG CAA CAC UAA ACG AGA AdTdT-3′) or WAVE2 (5′-GCA UCA AGC CUC UAA AGA AdTdT-3′) [20] were synthesized by RiboBio Co. Ltd. (Guangzhou, China). The siRNAs were transfected into the NSCLC cell line PG, respectively, with Lipofectamine™ 2000 (Invitrogen, USA). Effect of the knock-down was checked by Western blots immunoprobed with antibodies against HSPC300 or WAVE2, respectively.

2.5. Biological features of NSCLC cells with silenced HSPC300

2.5.1. Cell migration assays

PG cells were treated with HSPC300 siRNA for 24 h, then 1×10^5 cells were seeded into the upper chamber of a polycarbonate Transwell with an 8 μm-pore filter (Corning, Kennebunk, Maine, USA). Cells that had migrated across the filter over a 24-h period were fixed with 100% methanol, stained with 0.4% crystal violet, and counted under a microscope. The number of migratory cells per membrane was determined based on five field digital images taken randomly. Each determination represents the average of at least three individual wells.

2.5.2. Filamentous actin staining

Cells were washed with PBS (pH 7.4), fixed in methanol, penetrated with PBS containing 0.1% Triton X-100, blocked with 1% BSA, incubated with BODIPY FL phalloidin (Invitrogen, USA) for 30 min and then washed with PBS. FL phalloidin-stained cells were examined with a laser scanning confocal microscope (Leica, Wetzlar, Germany). Based on F-actin distribution and membrane structure, the cells were classified as: (a) polarized, with F-actin localized in pseudopodia; (b) unpolarized, with F-actin presenting as a circular structure in the cells; and (c) cells with intermediate features [21,22]. More than 100 cells were counted and categorized for each group

2.5.3. Scanning electron microscopy (SEM)

PG cells were fixed for 6 h in a solution containing 2.5% glutaraldehyde, 0.1 M cacodylate sodium buffer and 2% sucrose (pH 7.4). After washing with 0.1 M cacodylate buffer containing 2% sucrose, the cells were fixed with 1% OsO_4 in the washing buffer, dehydrated with ethanol, dried using a critical point drying method. Following sputter-coating with a 5–10 nm thick gold layer, the cell samples were examined employing a JSM-6000F SEM (JEOL, Tokyo, Japan)

2.6. Protein interaction between HSPC300 and WAVE2 in NSCLC cells

2.6.1. Immunoprecipitation and immunoblotting

PG cells were transfected with an expression plasmid encoding HSPC300 with a c-MYC tag (designated PHM) or the corresponding null vector (designated PV), respectively; and stable transfectants were selected. Cell lysates were prepared from the PHM and PV according to an established protocol [12], incubated with a monoclonal antibody against the c-MYC tag (Sigma, St. Louis, MO, USA) or an antibody against WAVE2 (Santa Cruz, USA), then precipitated with protein G-agarose beads (Invitrogen, USA). The resulting proteins were separated by Western blotting and detected with the antibodies against HSPC300, c-Myc, WAVE2 (BD Biosciences, San Jose, CA, USA), and β-actin (Sigma, USA), respectively.