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Filamin A upregulation correlates with Snail-induced epithelial to mesenchymal transition (EMT) and cell adhesion but its inhibition increases the migration of colon adenocarcinoma HT29 cells

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ABSTRACT

Filamin A (FLNA) is actin filament cross-linking protein involved in cancer progression. Its importance in regulating cell motility is directly related to the epithelial to mesenchymal transition (EMT) of tumor cells. However, little is known about the mechanism of action of FLNA at this early stage of cancer invasion.

Using immunochemical methods, we evaluated the levels and localization of FLNA, pFLNA[Ser2152], β1 integrin, pβ1 integrin[Thr788/9], FAK, pFAK[Y379], and talin in stably transfected HT29 adenocarcinoma cells overexpressing Snail and looked for the effect of Snail in adhesion and migration assays on fibronectin-coated surfaces before and after FLNA silencing.

Our findings indicate that FLNA upregulation correlates with Snail-induced EMT in colorectal carcinoma. FLNA localizes in the cytoplasm and at the sites of focal adhesion (FA) of invasive cells. Silencing of FLNA inhibits Snail-induced cell adhesion, reduces the size of FA sites, induces the relocalization of talin from the cytoplasm to the membrane area and augments cell migratory properties. Our findings suggest that FLNA may not act as a classic integrin inhibitor in invasive carcinoma cells, but is involved in other pro-invasive pathways. FLNA upregulation, which correlates with cell metastatic properties, maybe an additional target for combination therapy in colorectal carcinoma tumor progression.

1. Introduction

According to the statistics from 2012, colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. The invasive properties of neoplastic cells and their ability to form metastases are the leading causes of death in patients with this cancer type [1].

Epithelial to mesenchymal transition (EMT) is the critical mechanism by which epithelial-derived tumor cells become malignant and obtain an invasive phenotype. A large number of studies have found that metastatic cancer cells overexpress Snail-1 protein, a zinc-finger transcription factor which serves as one of the key inducers of EMT by affecting cell adhesion and migration [2,3]. Migrating cancer cells respond to various mechanical and chemical signals by modulating their cell shape or their adhesive properties.

Integrins, heterodimeric α- and β- chain surface receptors, are bidirectional transmembrane signaling receptors that mediate cell attachment to the extracellular matrix (ECM). They act as a platform for the recruitment of various cellular proteins, such as adaptor/scaffold and signaling proteins, to the inner plasma membrane surface, where they form structures called focal adhesions (FA) [4]. Most importantly, actin-binding proteins within FA such as talin, vinculin, FAK and the filamins are crucial for cell movement and adhesion to the ECM [5,6].

Filamin A (FLNA) is the most abundant and widely-expressed protein among the three filamin isoforms. It is a large (280 kDa) protein and a scaffolding molecule, regulating cell spreading and migration. As a mediator of cytoskeleton reorganization, it regulates FA disassembly at the leading edge of motile cells and localizes to the filopodia, lamellipodia, stress fibers and focal contacts [7,8]. FLNA also competes with talin, the main β-integrin activator protein, for an overlapping binding site on integrin, with the effect of inhibiting integrin activation [8–10]. During cell spreading, FLNA is concentrated near the cell membrane and adhesion sites [7]. FLNA proteolysis is regulated by the specific phosphorylation of the protein at serine

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residue 2152. Studies of various cancer types have suggested that FLNA expression and its susceptibility to cleavage may be regarded as a prognostic biomarker of cancer metastasis [11,12]; however, its exact role in the metastatic process remains elusive.

Previous studies on various cancers including those of the prostate or gastric tract have found FLNA upregulation and the presence of changes in its cellular localization to be associated with an invasive cell phenotype [9,11]. Therefore, the present study examines the level of FLNA expression and its function in adenocarcinoma cancer cells undergoing EMT, using a recently-described model of Snail-overexpressing human colorectal cancer cells [13–15].

2. Materials and methods

2.1. Reagents

All standard tissue culture reagents were purchased from Invitrogen (Eggenstein, Germany). Halt Protease Inhibitor Cocktail was obtained from Thermo Scientific Pierce, USA. Enhanced Chemiluminescence (ECL) Western blotting substrate and BCA Protein Assay Kit were purchased from Thermo Scientific Pierce (Minneapolis, USA). siRNA oligonucleotides were obtained from Dharmacon (USA) and the Nucleofection Kits from Lonza (Germany). Rabbit anti-Snail-1 and rabbit anti-vinculin antibodies were purchased from Cell Signaling (Danvers, USA). Mouse anti-filamin antibodies for western blotting (sc-271440), for C-terminus region of FLNA, recognizing 90 kDa fragment and total 280 kDa protein; goat anti-mouse antibodies, anti-goat and anti-rabbit antibodies conjugated with horseradish peroxidase were from Santa Cruz Biotech (Santa Cruz, CA). Phospho Safe Extraction Buffer, mouse anti-FLNA antibodies MAB 1680 clone TI10 (detecting both the 90 kDa fragment and the total 280 kDa protein) and rabbit anti-phospho-β1 integrin (Thr788/789) antibodies were purchased from Merck Millipore (Darmstadt, Germany). Goat anti-phospho-FLNA[Ser2152] (ab11074) antibodies as well as rabbit anti phospho-FAK [Y397], total FAK and MMP7 antibodies were from Abcam (USA). The FACS specific antibodies for alpha 2, alpha 4 and alpha 5 integrin receptors were obtained from BD Biosciences. The nitrocellulose membranes for western blotting were purchased from Bio-Rad, USA and Kodak BioMax Light films from Eastman Kodak. All other reagents, including fibronectin and collagen I proteins, were obtained from Sigma unless stated otherwise.

2.2. Cell culture

Adenocarcinoma cancer cell line HT29 was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in McCoy's 5 A, supplemented with L-glutamine (2 mM), 10% (v/v) FBS, streptomycin (100 μg/ml), and penicillin (100 units/ml). The cells were cultured as a monolayer at 37 °C in a humidified atmosphere with 5% CO_2. Stably transfected HT29 clones (HT29_Sn3 and HT29_Sn8) and empty vector transfected cells were previously characterized elsewhere [13–15].

2.3. Western blot assay

Briefly, exponentially growing cells were lysed with RIPA Reagent supplemented with Halt Protease Inhibitor Cocktail and the extracts were aliquoted and stored at −80 °C. They were then taken for protein quantification using the Bradford Protein Assay Kit (Bio-Rad, USA), according to the manufacturer's protocol. To detect the expression of phosphorylated FLNA, phosphorylated FAK and β1-integrin protein forms, the cells were washed with ice-cold PBS, and lysed with Phospho Safe Buffer at 4 °C, and treated as described before [16].

For pFAK and FAK detection, the cells were dissociated with trypsin diluted in full medium and seeded for 30 min at 37 °C in a humidified 5% CO_2 atmosphere on a 6-well plate coated with fibronectin (two hour-coating, 10 μg/ml/TBS, as described in Adhesion Assay). Then, the cells were washed with ice-cold PBS, and lyzed with Phospho Safe Buffer at 4 °C as described before.

Proteins (40 μg total) were separated on 4–10% SDS-PAGE gels and blotted (120 min, 200 mA, 4 °C) onto nitrocellulose membranes (Bio-Rad, USA). The membranes were then blocked for one hour with Tris-buffered saline (TBS) containing 5% BSA at room temperature and incubated with primary antibodies overnight at 4 °C. Then the membranes were washed, incubated for one hour at room temperature with horseradish peroxidase-conjugated secondary antibodies and further processed for chemiluminescence detection using Pierce ECL Western Blotting Substrate and Kodak BioMax Light Film. The developed films were scanned using an HP Scanjet G4050 scanner, and protein band intensity was quantified by ImageJ Software.

2.4. Filamin A silencing by RNA interference strategy

Specific (one of four) siRNA (sequence GAAUGGCGUUUACCUGAUU) targeting human FLNA and negative, non-targeting control siRNA were used. The siRNAs were transfected into HT-29 cells by electroporation according to the manufacturer's protocol (Nucleofection Assay, Lonza (Germany)). To silence FLNA, the siRNA treatment (200 nM) was performed for 72 h.

2.5. Migration assay

Tests were conducted on polycarbonate filters, 8 μm pore in diameter (Transwell Costar, USA). The lower compartment of the chamber was coated for two hours with 200 μl of fibronectin at 10 μg/ml/TBS. The cells were dissociated with trypsin and diluted in medium with 0.1% BSA to a final density of 2×10^6 cells/ml, and 50 μl of the cell suspension was added to the upper chamber. The assembled chambers were incubated for six hours at 37 °C and 5% CO_2. The cells on the upper surface of the filters were then removed with a cotton swab. The filters were fixed using cold 95% methanol/PBS for 5 min at room temperature. The cells were stained with Mayer's hematoxylin and eosin and washed in PBS. The cells from the “under surface” were collected, photographed and quantified with ImageJ Software.

2.6. Adhesion assay

Maxisorp loose Nunc-Immuno modules (Pittsburgh, PA, USA) were coated with 100 μl of 10 μg/ml fibronectin (Sigma-Aldrich, USA) or collagen I (Sigma-Aldrich, USA) for two hours. The wells were then washed and blocked for 1.5 h at 37 °C in a humidified 5% CO_2 atmosphere with 200 μl of 1% BSA/TBS (0.1 mM $CaCl_2$, 1 mM $MnCl_2$) following 1×10^5 cells/0.1 ml of medium for 1.5 h. The total amount of cell-associated protein was determined by dissolving the attached cells in 200 μl of BCA protein assay reagent directly in the microtiter wells. Absorbance was measured at 562 nm (Victor Spectrophotometer Reader, USA) [15,17].

2.7. Confocal microscopy

A total of 5×10^4 cells/ml was placed on sterile glass microscope slides and cultured at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. Cells with silenced FLNA expression were trypsinized after 72-h incubation, counted and placed on sterile glass microscope slides. They were then incubated for four hours at 37 °C in a humidified atmosphere of 5% CO_2 to allow them to attach to the surface.

The cells were washed with PBS, fixed with 4% formaldehyde in PHEM buffer (60 mM Pipes, pH 6.9, containing 25 mM Hepes, 10 mM EGTA, 4 mM $MgCl_2$ and protease inhibitors such as 1 mM PMSF, 0.1 mM EDTA, 1 mM leupeptin and 1 mg/ml aprotinin) for 20 min at room temperature.