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CA125/MUC16 interacts with Src family kinases, and over-expression of its C-terminal fragment in human epithelial cancer cells reduces cell–cell adhesion

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ABSTRACT

MUC16/CA125 is over-expressed in human epithelial tumors including ovarian, breast and some other carcinomas. The purpose of this study is to investigate how cell surface MUC16 is functionally involved in tumor progression, with a special focus on the role of its cytoplasmic tail. Forced expression of C-terminal MUC16 fragment (MUC16C) in epithelial cancer cells increased cell migration. We found that MUC16C directly interacted with Src family kinases (SFKs). Notably, localizations of E-cadherin and β-catenin at the cell–cell contacts were more diffuse in MUC16C transfectants compared with mock transfectants. Furthermore, MUC16C transfectants showed reduced Ca^{2+}-dependent cell–cell adhesion, but the treatment of cells with PP2, a SFKs inhibitor, restored this. Because cell surface MUC16 is also associated with the E-cadherin/β-catenin complex, the over-expression of MUC16 and its interaction with SFKs may enhance SFKs-induced deregulation of E-cadherin. Thus, our results suggest a role for cell surface MUC16 in cell–cell adhesion of epithelial cancer cells.

Introduction

MUC16 is a type I membrane glycoprotein comprising a long extracellular domain, a transmembrane domain and a short cytoplasmic domain (Bafna et al., 2010). This molecule is classified into the membrane-bound mucin family, and the extracellular domain is highly modified with a variety of N- and O-glycans (Kui Wong et al., 2003). It has been demonstrated that the glycoprotein carrying human ovarian cancer antigen CA125 is encoded by the *MUC16* gene (O'Brien et al., 2001; Yin and Lloyd, 2001). The serum levels of CA125/MUC16 (soluble MUC16 fragment, which is shed from cell surface) are elevated in >80% of patients with epithelial ovarian cancer (Bast et al., 1983). Furthermore, expression of CA125/MUC16 is also found in several epithelial carcinomas including pancreatic, breast, colon and lung cancers (Bast et al., 1998). The clinical utility and limitation of serum CA125/MUC16 as a cancer biomarker has been investigated in detail, but the biological functions of this mucin in tumor development have not been fully understood.

The membrane bound mucins such as MUC1 and MUC16 are generally present on the apical surface of normal epithelia. However, with transformation and loss of polarity, these mucins are often aberrantly over-expressed and distributed on the entire plasma membrane of epithelial cancer cells. There is accumulating evidence that MUC1, especially its cytoplasmic domain, is functionally involved in various oncogenic processes (Bafna et al., 2010; Kufe, 2009). For example, the cytoplasmic domain of MUC1 directly interacts with β-catenin, and competitively inhibits binding of β-catenin to E-cadherin (Li et al., 1998; Yamamoto et al., 1997). E-cadherin is concentrated at the adherens junction and plays an essential role in maintaining cell–cell adhesion of epithelial cells. β-catenin links E-cadherin to the actin cytoskeleton by interacting with α-catenin. The loss/reduction of E-cadherin expression or function is thought to be a key step of epithelial-to-mesenchymal transition of epithelial carcinomas (Thiery et al., 2009). Notably, it has been reported that MUC16 is associated with the E-cadherin/β-catenin complex (Comamala et al., 2011). This finding indicates that over-expressed MUC16 may also be involved in regulation of E-cadherin-mediated cell–cell adhesion in epithelial cancer cells. Unlike MUC1, however, the cytoplasmic domain of MUC16 does not contain the consensus sequence responsible for direct binding to β-catenin.

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The cytoplasmic domain of MUC16 is composed of 35 amino acids. As a functional motif, this domain contains a polybasic amino acid sequence (RRRKK), which interacts with ezrin/radixin/moesin (ERM) proteins. ERM proteins have been thought to function as cross-linkers between cell surface molecules and actin filaments (Tsukita and Yonemura, 1999). Therefore, it has been suggested that the cell surface MUC16 may be linked to the actin cytoskeleton through interaction with ERM proteins (Blalock et al., 2007). Janus kinase-2 (JAK2) possesses an ERM domain at the N-terminus. Recently, association of JAK2 with MUC16 has been shown to activate STAT3 and c-Jun, and to promote cell proliferation of breast cancer cells (Lakshmanan et al., 2012).

In the present study, we demonstrate that the cytoplasmic domain of MUC16 directly interact with SFKs such as c-Src and c-Yes. Furthermore, our results suggest that this molecular interaction may be involved in regulation of E-cadherin-mediated cell–cell adhesion.

Materials and methods

Cell culture

HCT116 (a human colon cancer cell line) and MCF7 (a human breast cancer cell line) cells were maintained in DMEM medium containing 10% heat-inactivated fetal bovine serum (HI-FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. OVCAR3 (a human ovarian cancer cell line) cells were cultured in RPMI1640 medium containing 20% HI-FBS, 1 mM sodium pyruvate, 10 μg/ml bovine insulin, 100 units/ml penicillin and 100 μg/ml streptomycin. These cells were obtained from the American Cell Type Collection.

Antibodies and reagents

Anti-phospho Akt (Ser-473) polyclonal antibody (pAb), anti-Akt pAb, anti-phospho ERK1/2 (Thr-202/Tyr-204) monoclonal antibody (mAb), anti-ERK pAb, anti-phospho c-Src (Tyr-416) pAb, anti-c-Src mAb, anti-E-cadherin mAb, and LY294002 were purchased from Cell Signaling Technology (Beverly, MA). Anti-β-actin mAb, anti-FLAG mAb, sodium orthovanadate and PP2 were purchased from Sigma-Aldrich (St. Louis, MO). Anti-c-Yes pAb and PP3 were purchased from Millipore (Bedford, MA). Anti-β-catenin mAb and anti-phosphotyrosine mAb PY99 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Expression vector

Using Isogen reagent (Nippon Gene, Tokyo, Japan), total RNA was prepared from OVCAR3 cells. First-strand cDNA was synthesized with the ReverTra Ace® cDNA synthesis reagents (TOYOBO, Tokyo, Japan). 1 μg of total RNA, 1 μl of random oligo(dT) primer (10 pmol/μl), 4 μl of 5× reaction buffer, 1 μl of ribonuclease inhibitor (10 U/μl) and 2 μl of dNTP mixture (10 mM each) were mixed and incubated at 42 °C for 30 min. The reaction was stopped by heating at 99 °C for 5 min. PCR was carried out at 25 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 s and extension at 72 °C for 2 min. 20 μl of the reaction mixture contained 1 μl of cDNA, 2 μl of 10× reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 U of LA-Taq-polymerase (TaKaRa, Kyoto, Japan), and 0.2 μM forward (5′-GCAAGGGCTCAGCTACATTC-3′) and reverse (5′-CCAGCCAAGCTTCTTTGGAC-3′) primers. The PCR product was cloned into pCR2.1-TOPO vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), sequenced and then subcloned into pFLAG-CMV™-3 expression vector (Sigma–Aldrich). Site-directed mutagenesis was performed with the QuickChange XL kit (Stratagene, La Jolla, CA).

Cell transfection

Two μg of expression plasmids was transfected into cells using FuGENE HD transfection reagent (Promega, Madison, WI). Stable transfectants were selected with G418 (600 μg/ml). Expression levels of FLAG-tagged MUC16C were examined by fluorescence-activated cell sorting (FACS) analysis using an anti-FLAG mAb (Supplemental Fig. 1D–G). Based on higher levels of cell surface MUC16C, two independent clones were established for each stable transfection, and were characterized in this study. For the transient transfection experiments, cells were used after 2 days of transfection.

Preparation of cell extract and immunoprecipitation

Cells were solubilized with 2% SDS, and then sonicated for 5 min. Protein concentrations were determined by the Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amount of proteins (5–10 μg protein) were analyzed by western blotting. For immunoprecipitation, cells were solubilized with cell extraction buffer (25 mM Tris–HCl, pH 7.5 buffer containing 0.15 M NaCl, 1% TX-100, 100 μM pervanadate, phosphatase and protease inhibitor cocktails) (Nacalai Tesque, Kyoto, Japan). After centrifugation at 13,000 × *g* for 10 min at 4 °C, the supernatant was collected, and then incubated with 20 μl of anti-FLAG mAb-affinity gel (Sigma–Aldrich) overnight at 4 °C. After washing four times with cell extraction buffer, immunoprecipitates were analyzed by western blotting.

Western blotting

Cell lysates and immunoprecipitates were separated by SDS-PAGE (8%), and transferred to polyvinylidene fluoride membranes. After blocking with Tris–HCl, pH7.5 buffer containing 0.15 M NaCl and 5% BSA or 4% BlockAce (Dainippon Pharmaceutics, Osaka, Japan), the membranes were incubated with primary antibodies; anti-Akt pAb (1:1000 dilution), anti-phospho Akt (Ser-473) pAb (1:1000 dilution), anti-ERK1/2 pAb (1:1000 dilution), anti-phospho ERK1/2 (Thr-202/Tyr-204) mAb (1:2000 dilution), anti-c-Src mAb (1:1000 dilution), anti-phospho c-Src (Tyr-416) pAb (1:1000 dilution), anti-c-Yes pAb (1:500 dilution), anti-phosphotyrosine mAb PY99 (1:200 dilution), anti-E-cadherin mAb (1:1000 dilution), anti-β-catenin mAb (1:200 dilution) and anti-β-actin mAb (1:5000 dilution). Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and chemiluminescence reagents. For the detection of FLAG-tagged MUC16C recombinant proteins, the membranes were incubated with HRP-conjugated anti-FLAG mAb (1:20,000 dilution). The intensities of the bands were measured using the ImageJ program.

Cell migration assay and MTT assay

Cell migration ability was evaluated *in vitro* using the transwell chambers (24-well culture plate) with an 8.0 μm pore polycarbonate membrane insert (Corning Inc., Corning, NY). The bottom of membrane filter was pre-coated with 1 μg of fibronectin. Briefly, cells (4×10^4) in DMEM supplemented with 0.2% BSA were seeded on the upper wells. The lower wells were filled with DMEM containing 10% HI-FBS. In some cases, chemical inhibitors were added into both upper and lower wells. After incubation for 20 h, non-migrated cells on the top of filter were removed with a cotton swab, and then migrated cells on the bottom of filter were fixed with methanol, stained with Diff-Quick. The numbers of migrated cells in five randomly selected fields were counted. For MTT assay,