Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 408–416
BBRC
www.elsevier.com/locate/ybbrc

CD151 forms a functional complex with c-Met in human salivary gland cancer cells ☆

Sebastian Krystian Klosek [a], Koh-ichi Nakashiro [a,*], Shingo Hara [a], Satoru Shintani [a], Hitoshi Hasegawa [b], Hiroyuki Hamakawa [a]

[a] *Department of Oral and Maxillofacial Surgery, Ehime University School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan*
[b] *First Department of Internal Medicine, Ehime University School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan*

Received 11 August 2005
Available online 24 August 2005

Abstract

In this study, we have attempted to elucidate the expression and function of CD151 in human salivary gland cancer cells. CD151 expression was detected in Acc2 and AccM cells, but not in normal tissues and primary cultured epithelial cells derived from human salivary gland. CD151 has been found to function as a molecular linker in the formation of complexes between c-Met/hepatocyte growth factor (HGF) receptor and integrin α3/α6. Knockdown of CD151 or integrin α3/α6 expression almost completely abrogated HGF-stimulated cell growth and migration. In contrast, forced expression of CD151 in Acc2 cells resulted in the increase of the HGF-dependent biological effects. These results suggest that CD151 forms a structural and functional complex with c-Met and integrin α3/α6, and exerts its oncogenic functions through excessive activation of the HGF/c-Met signalling pathway.

Keywords: CD151; c-Met; HGF; Integrin α3/α6; Salivary gland cancer

CD151, a member of the tetraspanin family, has been shown to influence various biological processes including cell motility, adhesion, and morphogenesis, and it is also known to be associated with the cytoskeleton [1–5]. Tetraspanin-induced cell migration and rapid metastasis have been observed in many human malignancies [6–9]. In addition, functional connections have been reported between CD151 and some integrin subunits, of which the most important are the laminin receptors. The CD151 and integrin α3/α6 complexes function during cellular morphogenesis as components of integrin signalling [3,4,10]. The CD151 and integrin α3β1 complex is a component of the cadherin–catenin complex which regulates cell–cell adhesion [2] and reorganization of the actin cytoskeleton [11], and is involved in the adhesion-dependent production of matrix metalloproteinase-2 [12]. CD151/integrin α6β4 is a constituent of hemidesmosomes and helps to maintain their spatial organization [5]. CD151 has also been described in connection with α4β1, α5β1, α7β1, and IIbβ3 integrins [10,13–15]. Recent studies have demonstrated that tetraspanins form structural and functional associations with tyrosine kinase receptors. CD82 has been reported to attenuate signalling and regulate compartmentalization and the ligand-induced dimerization of epidermal growth factor receptor (EGFR) [16,17]. CD9 is thought to influence transforming growth factor-α release, EGFR activation, and cell proliferation [18], as well as CD9 has the capacity to associate with the membrane-anchored heparin-binding epidermal growth factor-like precursor/diphtheria toxin

☆ *Abbreviations:* BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FITC, isothiocyanate fluorescein; GFP, green fluorescent protein; HGF, hepatocyte growth factor; IFA, indirect immunofluorescence assay; MFI, median fluorescence intensity; PBGD, porfobilinogen deaminase; PBS, phosphate-buffered saline; PI, propidium iodide; RNAi, RNA interference; SDS, sodium dodecyl sulfate; SG, salivary gland; SGEC, primary cultured epithelial cells; siRNA, small interfering RNA; WHA, wound healing assay.

* Corresponding author. Fax: +81 89 9605396.
E-mail address: nakako@m.ehime-u.ac.jp (K. Nakashiro).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.106

receptor [19]. Hepatocyte growth factor (HGF), which was originally identified as a potent mitogen that stimulates the growth of hepatocytes [20], is a pleiotropic polypeptide growth factor that regulates cell proliferation, migration, survival, angiogenesis, and invasion [21–23]. These diverse biological effects are mediated through its receptor, which is expressed by a proto-oncogene known as *c-met*. The interaction between c-Met and integrin α6β4 is essential for HGF-dependent invasive growth [24]. Since the previously reported functions of CD151 and c-Met appear very similar, we investigated the possibility of interaction between CD151 and c-Met, and its direct participation in ligand-induced signal transduction. In the present study, we used two human salivary gland cancer cell lines, Acc2 and AccM, which arose from the same origin but are characterized by different growth and metastatic potency [25,26].

Materials and methods

Cells and cell culture. Human salivary gland adenoid cystic carcinoma cell lines (AccM and Acc2) were kindly provided by Dr. W.L. Qiu [25,26]. Cells were cultured under standard culture conditions in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Biosource International, Camarillo, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Tissue samples. Normal submandibular glands were obtained from patients undergoing treatments at Ehime University School of Medicine (Toon, Japan) and were designed for protein extraction procedures and establishment of primary cultured epithelial cells.

Antibodies. The primary antibodies used were as follows: anti-CD151 14A2.H1 (Research Diagnostics, Flanders, NJ.); anti-Met (C-12), anti-α3 (I-19) integrin, anti-α6 (N-19) integrin, and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies used were as follows: anti-rabbit IgG or anti-mouse IgG (Amersham Biosciences, Piscataway, NJ); anti-goat IgG (Santa Cruz Biotechnology); anti-mouse Alexa488-conjugated IgG (Molecular Probes, Eugene, OR); anti-mouse fluorescein isothiocyanate (FITC)-labelled IgG + IgM (Biosource); and anti-rabbit Alexa568- and Alexa546-labelled IgG (Molecular Probes).

Small interfering RNAs. Cells were transfected with small interfering RNAs (siRNAs) specific for CD151 (CD151-siRNA; Smartpool siRNA reagents, Cat. No. M-003637-00-05, Dharmacon, Lafayette, CO), integrin α3 (α3-siRNA; Smartpool siRNA reagents, Cat. No. M-004571-00) and α6 (α3-siRNA; Smartpool siRNA reagents, Cat. No. M-007214-00), and green fluorescent protein (GFP-siRNA; Smartpool siRNA reagents, Cat. No. D-001300-01-20, Dharmacon) as a control, at a concentration of 10 nM using lipofection (Lipofectamine 2000; Invitrogen, Life Technologies) according to the manufacturer's instructions. After 48 h, gene-knockdown cells were recovered by treatment with 0.05% trypsin–0.53 mM EDTA (Invitrogen) and used for the cell growth assay, immunoprecipitation, and wound healing assay (WHA).

Cell growth assay. Cells were seeded in plain medium on plastic six-well plates or ready-to-use six-well plates coated with Matrigel (100 μg/cm^2, Biocoat Cell Environments; BD Biosciences, Bedford, MA) at a density of 5×10^4 cells per well. After 24 h, culture medium was changed by growth medium or plain RPMI 1640 with or without HGF (40 ng/ml; R&D Systems, Minneapolis, MN). The cells were recovered by treatment with 0.05% trypsin–0.53 mM EDTA (Invitrogen) and counted using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA). GFP-siRNA transfected cells were used as controls.

Wound healing assay. siRNA-transfected cells were seeded on plastic six-well plates or ready-to-use culture plates coated with Matrigel and grown for 24 h to confluence. The medium was replaced with fresh plain RPMI 1640 medium 12 h before the onset of the assay. The cell monolayers were scratched with a pipette tip to make a wound that was 0.5 mm wide. The wounded cells were incubated in growth medium or in plain RPMI 1640 medium with or without HGF (40 ng/ml; R&D systems) for 24 h.

Immunoprecipitation and Western blot analysis. Western blot analysis was done as described previously [23]. Protein A and Protein G immunoprecipitation kits (Roche Diagnostics) were used for the immunoprecipitation, and the immunoprecipitates were prepared according to the manufacturer's instructions.

Immunofluorescence staining. The cells were grown overnight on glass coverslips in culture dishes. Later cells were washed, fixed in 2% formaldehyde, and blocked with 10% bovine serum albumin (BSA). After blocking, the cells were incubated with CD151 and c-Met antibodies in 0.1% saponin + 10% BSA/PBS (phosphate-buffered saline) in moist chambers for 1 h, followed by anti-mouse Alexa488-labelled IgG and anti-rabbit Alexa568-labelled IgG (Molecular Probes) under the same conditions. Isotype controls for 14A2.H1 antibody followed by Alexa488-conjugated antibody were used. 4′,6-diamidino-2-phenylindole (DAPI; Wako, Osaka, Japan) was used to stain the nuclei. Finally, the samples were mounted in Prolong-Antifade (Molecular Probes) and examined using a fluorescence microscope (E800; Nikon, Japan) or a confocal microscope (C-1; Nikon, Japan).

Flow-cytometric analysis. Cells (1×10^7 per sample) were fixed in 2% formaldehyde, blocked in 10% BSA solution, and stained with antibodies against CD151 and c-Met in 0.1% saponin/PBS. This was then incubated with the FITC-conjugated anti-mouse IgG (isothiocyanate fluorescein) and Alexa546-labelled anti-rabbit IgG secondary antibodies. Isotype controls for 14A2.H1 antibody followed by FITC-conjugated antibody were used. Analysis was performed using a FACScan flow cytometer (FACSCalibur) with the CellQuest software (BD Biosciences).

For the analysis of cell cycle, the harvested cells (floating in medium and attached to the culture dish bottom) were centrifuged at 500*g* for 5 min. Thereafter, cells were washed once with PBS. Then cell suspension was centrifuged again and resuspended in 70% ethanol. After incubation at −20 °C for at least 24 h, the cells were washed in 1 ml PBS and resuspended in 0.25 ml ribonuclease A (RNase A, Wako) solution followed by incubation at 37 °C for 15 min. Isotonic propidium iodide (PI, Sigma) was added and cells were incubated at room temperature for 15 min in darkness. PI was excited at 488 nm, and fluorescence signal was subjected to logarithmic amplification with PI fluorescence (red) being detected above 600 nm.

Plasmid and transfection. Cultured Acc2 cells were transfected with the plasmid pL2neoSRαIIISFA-1 containing sense sequence of the CD151 tetraspanin [1,6]. Lipofection method (Lipofectamine 2000, Invitrogen) was used according to the manufacturer's instructions. For stable transfectants of Acc2 cells, the cells were passaged into fresh growth medium 24 h after transfection and cultured medium was supplemented with 0.8 mg/ml G418 Disulfate Aqueous Solution (Nacalai Tesque, Kyoto, Japan) the following day and thereafter.

For stable transfectants of GFP-AccM cells, transfection and clone isolation were performed as described above with the vector pEGFP-N3 (BD Biosciences, Clontech) containing sense sequence of the GFP.

RNA isolation and real-time quantitative RT-PCR. Total RNA was extracted using the RNA isolation solution, Isogen (Nippone Gene, Tokyo, Japan), according to the manufacturer's instructions and subjected to cDNA synthesis and PCR amplification. Reactions were performed using a LightCycler RNA Amplification Kit Hybridization Probes (Roche Molecular Biochemicals). The primer and TaqMan probe sequences for CD151 gene were as follows: forward primer, 5′-CGC TGG TAT CCT CGC CT-3′; reverse primer, 5′-CCT TCA GGT TCT CCT TGA GCT-3′, and TaqMan probe, 5′-CGC CTA CTA CCA GCA GCT GAA CAC G-3′ (Geneset Oligos, La Jolla, CA). For quantification of porfobilinogen deaminase (PBGD) as an internal control gene the following sequences were used: forward primer, 5′-TGT CTG GTA ACG GCA ATG C-3′; reverse primer, 5′-CGC GAA TCA CTC TCA TCT TTG-3′, and TaqMan probe, 5′-TGC AAC GGC GGA AGA AAA CAG C-3′ (Geneset Oligos).