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mTORC2 activation is regulated by the urokinase receptor (uPAR) in bladder cancer☆

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ABSTRACT

Mammalian target of rapamycin complex 2 (mTORC2) has been identified as a major regulator of bladder cancer cell migration and invasion. Upstream pathways that mediate mTORC2 activation remain poorly defined. Urokinase-type plasminogen activator receptor (uPAR) is a GPI-anchored membrane protein and known activator of cell-signaling. We identified increased uPAR expression in 94% of invasive human bladder cancers and in 54–71% of non-invasive bladder cancers, depending on grade. Normal urothelium was uPAR-immunonegative. Analysis of publicly available datasets identified uPAR gene amplification or mRNA upregulation in a subset of bladder cancer patients with reduced overall survival. Using biochemical approaches, we showed that uPAR activates mTORC2 in bladder cancer cells. Highly invasive bladder cancer cell lines, including T24, J82 and UM-UC-3 cells, showed increased uPAR mRNA expression and protein levels compared with the less aggressive cell lines, UROtsa and RT4. uPAR gene-silencing significantly reduced phosphorylation of Serine-473 in Akt, an mTORC2 target. uPAR gene-silencing also reduced bladder cancer cell migration and Matrigel invasion. S473 phosphorylation was observed by immunohistochemistry in human bladder cancers only when the tumors expressed high levels of uPAR. S473 phosphorylation was not controlled by uPAR in bladder cancer cell lines that are PTEN-negative; however, this result probably did not reflect altered mTORC2 regulation. Instead, PTEN deficiency de-repressed alternative kinases that phosphorylate S473. Our results suggest that uPAR and mTORC2 are components of a single cell-signaling pathway. Targeting uPAR or mTORC2 may be beneficial in patients with bladder cancer.

1. Introduction

Bladder cancer is the fifth most common cancer, affecting more than half a million people in the United States [1]. Invasive high-grade urothelial carcinoma accounts for the majority of bladder cancer deaths; the 5-year survival is 34% for patients with locally advanced disease and 6% for patients that develop distant metastases [1]. A deeper understanding of the molecular mechanisms that drive invasive behavior in bladder cancer is important. Our group previously identified mammalian target of rapamycin complex 2 (mTORC2) as a critical regulator of bladder cancer invasion [2].

mTORC2 is a complex of proteins associated with the serine-threonine kinase, mTOR, that includes Rictor, mLST8, mSIN1, and Protor. mTORC2 is distinguished from the second mTOR-containing complex, mTORC1, by subunit composition and kinase activity. Although mTORC2 is a major regulator of cellular metabolism and the cytoskeleton, pathways involved in mTORC2 activation remain incompletely understood. mTORC2 may be activated by phosphatidylinositol-3,4,5-triphosphate (PIP_3) [3], suggesting that PI3K plays an essential role [4]. mTORC2 also may be activated by associating with ribosomes [5]. An important biomarker of mTORC2 activation is phosphorylation of Serine 473 in Akt, which is necessary for optimal activation of Akt [6]. However, other enzymes, termed PDK2s, also may phosphorylate S473, including integrin-linked kinase (ILK) [7], protein kinase C (PKC)α [8], PKC-βII [9], MAPK kinase (MK)2 (10), DNA-dependent kinase (DNA-PK) [11], ataxia telangiectasia mutated (ATM) [12], p21-activated kinase (PAK)1, and PAK2 [13].

mTORC2 is activated in muscle-invasive bladder cancer and selective targeting of mTORC2 abrogates bladder cancer cell invasion by decreasing Rac1 activation [2]. The goal of the present study was to identify signaling systems that may function upstream of mTORC2 in bladder cancer. Phosphatase and tensin homolog on chromosome 10 (PTEN) is a protein phosphatase, which may be deleted or mutated in bladder cancer [14]. Given the role of PTEN in regulating PIP_3, we hypothesized that this gene product may regulate mTORC2 activity. The urokinase receptor (uPAR) is a GPI-anchored receptor that forms a multiprotein

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complex to activate important cell-signaling factors that support cancer cell migration and invasion, including Rac1 [15,16]. uPAR also promotes epithelial-mesenchymal transition [17] and may confer stem cell-like properties in cancer cells [18].

In contrast with normal human adult tissues, which demonstrate low levels of uPAR expression, uPAR is frequently up-regulated in invasive cancers and is correlated with poor clinical outcomes [19]. Prior studies demonstrated increased soluble uPAR in the urine of patients with invasive bladder cancer and an increase in uPAR protein in invasive bladder cancers [20,21]. Although these results suggest that uPAR may affect the course of bladder cancer, molecular mechanisms by which uPAR regulates bladder cancer cell physiology remain undefined.

In the current study, we show that uPAR is upregulated in invasive human bladder cancer and in highly invasive urothelial carcinoma cell lines. We further show that uPAR is a major regulator of mTORC2 activation. PTEN deficiency also was associated with robust phosphorylation of Akt S473; however, this effect did not reflect mTORC2 activation but instead, activation of alternative PDK2s. Silencing uPAR gene expression inhibited bladder cancer cell migration and invasion, irrespective of whether the bladder cancer cell line expressed PTEN. These findings suggest that uPAR may be a novel regulator of pro-invasive cell-signaling in bladder cancer and may represent a new therapeutic target in this disease.

2. Materials and methods

2.1. Cell culture and reagents

RT4, UM-UC-3, T24, J82 and J82 (clone J82C) bladder cancer cells, U87-MG and U251-MG high grade glioma (HGG) cells, and MDA-MB-231 and MDA-MB-468 breast cancer cells, PANC-1 pancreatic cancer cells, and PC-3 prostate cancer cells were purchased from the American Type Tissue Collection (ATCC, Manassas, VA, USA). U251-MG cells were referred to as U373-MG cells in previous publications [22]. UROtsa cells were obtained from Prof. Alice Fabarius (University of Heidelberg, Mannheim, Germany) and were previously authenticated by STR analysis by the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures) [23]. Authentication of all other cell lines by STR analysis was performed by IDEXX BioResearch (Columbia, MO, USA). Bladder cancer cells were cultured in RPMI-1640 media (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). HGG, MDA-MB-468, PANC-1 and PC-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS. MDA-MB-231 cells were cultured in Leibovitz-15 (Gibco) medium supplemented with 10% FBS. All cell lines were maintained at 37 °C in a humidified chamber containing 5% CO_2. All experiments with cell lines were performed within 20 routine passages after thawing from cryopreservation.

Mouse anti-uPAR (clone 62022) and anti-urokinase-type plasminogen activator (uPA) (clone 204212) antibodies were purchased from R&D Systems (Minneapolis, MN, USA; 1:1000 dilution). Antibodies targeting Rictor (1:1000; cat. # 2140), phospho-Akt S473 (1:1000; cat. # 4060), total Akt (1:1000; cat. # 4691), plasminogen activator inhibitor-1 (PAI-1) (1:1000; cat. # 11907), and PTEN (1:1000; cat. # 9559) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody specific for actin (1:5000; cat. # 2066) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Patient specimens and immunohistochemistry

Bladder cancer specimens were obtained from patients with normal urothelium, low-grade papillary urothelial carcinoma, high-grade papillary urothelial carcinoma, and invasive urothelial carcinoma. Variant morphologies were excluded. Tissue analysis without patient identifiers was approved by the University of California at San Diego Institutional Review Board. Tissue microarrays were generated from formalin-fixed, paraffin-embedded blocks. At least 4 tissue core punches were obtained from each normal or cancer specimen. The mean patient age was 65.1 (range 43 to 84 years). The male:female ratio was 2.4 to 1.

Tissue sections (4 μm) were transferred to pre-coated slides. Deparaffinization, rehydration, and heat-induced antigen retrieval using sodium citrate buffer were performed. Sections were blocked with 10% normal goat serum (Cell Signaling Technology) in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated overnight at 4 °C with monoclonal primary antibody against human uPAR (clone R4; Dako North America, Inc., Carpinteria, CA, USA), PTEN (clone D4.3; Cell Signaling), or phospho-Akt S473 (clone DE9; Cell Signaling) in blocking buffer. The next day, slides were washed 4 times in TBS. Blocking of endogenous peroxidase was performed by incubation with 3% H_2O_2 in TBS for 10 min. The slides were then incubated with HRP polymer secondary antibody and 3,3′-diaminobenzidine (DAB) using an HRP/DAB (ABC) Detection IHC Kit according to manufacturer instructions (AbCam, Cambridge, MA, USA). Slides were reviewed using an Olympus BX-40 microscope (Waltham, MA) and images captured using an Olympus DP22 camera.

2.3. Gene silencing

siRNA specific for uPAR mRNA (custom sequence: 5′–GCCGUUACCUCGAAUGCAU dT dT–3′), Rictor siRNA (cat. # M-016984-02), PTEN siRNA (cat. # M-003023-02) and non-targeting control (NTC) siRNA #3 (cat. # D-001210-03) were from GE Dharmacon (Lafayette, CO, USA). Unless otherwise noted, transfections were performed for 48 h using Lipofectamine® RNAiMAX (Invitrogen) reagent according to the manufacturer's instructions.

2.4. Immunoblotting

Whole cell extracts were prepared using radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) containing complete protease and PhosSTOP phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN, USA). Conditioned serum-free medium (SFM) was collected from cell cultures that were 70–80% confluent, following incubation for 16–18 h. Conditioned medium (CM) samples were concentrated using Amicon Ultra-4 Centrifugal Filter Units with Ultracel-10 membranes (EMD Millipore, Billerica, MA, USA). Protein content in cell extracts was determined by BCA assay. Equal amounts of cellular protein (5–10 μg) or equal volumes of concentrated CM were separated by SDS-PAGE. Proteins were electrotransferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (0.45 μm; EMD Millipore). Membranes were blocked in 5% (w/v) non-fat dry milk (Kroger, Cincinnati, OH, USA) in TBS with 0.1% Tween-20 (TBS-T) for 1 h and then incubated overnight at 4 °C with appropriate primary antibodies in 5% (w/v) bovine serum albumin (BSA) in TBS-T. Membranes were washed in TBS-T and incubated with HRP-conjugated secondary antibody (dilution range 1:5000–1:30,000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Secondary antibody was detected using Clarity™ ECL Western Blotting substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Densitometry analysis was performed with ImageJ software (version 1.48).

2.5. Real-time qPCR

Total RNA extraction and real-time qPCR was performed as previously described [24]. TaqMan probes for human uPAR (Hs00958880_m1), uPA (Hs01547054_m1), PAI-1 (Hs01126606_m1), and GAPDH (Hs02758991_g1) were purchased from Life Technologies (Thermo Fisher Scientific). Relative mRNA expression values were calculated using the ΔC_t method and normalized to GAPDH mRNA.