SH3BGRL3 Protein As A Potential Prognostic Biomarker For Urothelial Carcinoma: A novel binding partner of epidermal growth factor receptor

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Disclosure of Potential Conflicts of Interest

All authors declare no conflict of interest.

Translational Relevance

Our prior studies indicate that EGFR, whether by itself or co-expressed with ErbB2, ErbB3 or RON, is an important predictor of tumor recurrence and survival of urothelial cancer patients. The data support the importance of EGFR in the tumorigenesis of human bladder. We report that detectable urinary SH3BGRL3 is positively associated with histologic grading and muscle-invasiveness of urothelial carcinoma. Higher SH3BGRL3 expression in urothelial carcinoma is related to increased risk of recurrence and lower survival rate. SH3BGRL3 promotes the cell growth in vitro and in vivo. Evidence provided further supports activation of Akt-associated signaling cascade by SH3BGRL3 and its interaction with EGFR by proline-rich residues through Grb2. Hence, we have unveiled that SH3BGRL3 is a novel urinary biomarker for urothelial carcinoma. SH3BGRL3 deserves investigation as a co-targeting candidate in the design of EGFR-based cancer therapy.
Abstract

Purpose: Mass spectrometry-based biomarker discovery has clinical benefit. To identify novel biomarker for urothelial carcinoma (UC), we performed quantitative proteomics on pooled urine pairs from patients with and without UC.

Experimental Design: Shot-gun proteomics using LC-MS/MS and stable isotope dimethyl labeling identified 219 candidate proteins. The potential implication of SH3 domain binding glutamic acid-rich protein like 3 (SH3BGRL3) was examined by immuno-blotting of the urine (n = 13) and UC tumors (n = 32). Additional immunohistochemistry was performed on bladder cancer array (n = 1145) and correlated with tumor aggressiveness. Then, biological functions and signaling pathways of SH3BGRL3 were explored using stable cell lines.

Results: The detectable urine SH3BGRL3 in UC patients was positively associated with higher histologic grading and muscle-invasiveness of UC. SH3BGRL3 is expressed in 13.9% (159/1145) of bladder cancer cohort and is positively associated with muscle invasion (p = 0.0028). SH3BGRL3 expression is associated with increased risk of progression in patients with non-muscle-invasive bladder cancer (p = 0.032). SH3BGRL3 expression is significantly associated with high level of epidermal growth factor receptor (EGFR) in bladder cancer (p < 0.0001). SH3BGRL3 promotes the epithelial-mesenchymal transition, cell migration, and proliferation of UC in vitro. SH3BGRL3 interacts with phosphor-EGFR at Y1068, Y1086 and Y1173 through Grb2 by its proline-rich motif, and activates Akt-associated signaling pathway.

Conclusions: Evaluation of SH3BGRL3 expression status or urine content may identify a
subset of bladder cancer patients who may require more intensive treatment. SH3BGRL3 deserves further investigation as a co-targeting candidate for designing EGFR-based cancer therapies.

Keywords: SH3BGRL3, EGFR, Akt, Urothelial Carcinoma
Introduction

Urothelial carcinoma (UC) of the bladder is the 6th most common human cancer in the United States, with 74,000 new cases and 14,000 cancer deaths estimated in 2015 (1). UC of the upper urinary tract (UC-UUT) is rare, representing only 5% of all UC (2). It is well-known that UC is fundamentally multifocal in nature: in patients with bladder cancer, 4% will eventually develop UC-UUT, while up to 50% of those with UC-UUT will develop a lesion in the bladder (3). The established prognostic factors for patients with UC are age, tumor staging and histological grade (3, 4). Therefore, it is necessary to identify biomarkers that are useful for diagnosis and predicting the prognosis when planning treatment for UC.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics has become a popular tool for studying biomarker. Stable-isotope labeling coupled with shot-gun proteomics can explore the protein profile of a biological sample in a single run of MS and distinguish protein expression levels between two biological states (5). We have developed a stable isotope-based dimethyl labeling method that produces different isotopic pairs ranging from 2 to 8 Da (mass difference) or up to 6 multiplex analyses (6). By using stable isotope dimethyl labeling coupled with two-dimensional liquid chromatography peptide separation and MS/MS analysis, we discovered that nucleophosmin is positively involved in the arsenic-related bladder carcinogenesis (7). The urine profiling experiment found that CD14 is a potential biomarker for benign prostatic hyperplasia (8). The results support the accuracy and clinical implications of this technical platform.
SH3 domain binding glutamic acid-rich protein like 3 (SH3BGRL3), one of candidate proteins in the gene list, is located on chromosome 1p34.3-35. It contains 279 nucleotides that are translated to 93 amino acids. The crystal and NMR structures of SH3BGRL3 have been described in an earlier work (9). The amino acid sequence of SH3BGRL3 belongs to the thioredoxin superfamily, and is highly similar to *E. coli* GRX1 (10), an enzyme with oxidoreductase activity. Although SH3BGRL3 protein is homologous to N-terminal region of the SH3BGR protein (10, 11), it lacks the conserved SH3-binding motif, implying that it has a different function to those of other subfamily members. SH3BGRL3 could inhibit TNF-α-induced apoptosis and promote cell survival, although the molecular mechanism underlying these functions remains unclear (12). In a pig-human xenograft model experiment, SH3BGRL3 was up-regulated in xeno-conjunctival epithelial cells to protect them from programmed cell death (13). SH3BGRL3 is also involved in all-trans retinoic acid-induced cell differentiation (9).

Here, we describe the detection of SH3BGRL3 in the urine of patients with UC and up-regulated SH3BGRL3 in primary bladder cancer. Further investigations revealed that SH3BGRL3 participates in cancer cell proliferation *in vitro* and *in vivo*, EMT, and cell migration. Of particular importance, we provide evidence that SH3BGRL3 interacts with epidermal growth factor receptor (EGFR) at Y1068, Y1086 and Y1173 through Grb2 by its proline-rich motif, and activates the Akt-associated signaling pathway. Our results suggest that SH3BGRL3 is a novel co-targeting candidate for EGFR-based cancer therapy.
Materials and Methods

Patient enrollment and sample and data collection

A total of 1190 patients were enrolled and all of the participants with radical cystectomy had signed informed consent forms before the study began. The study protocol was approved by the Institutional Review Board of National Cheng Kung University Hospital (No.HR-97-117 and B-ER-103-214) and Taipei Veterans General Hospital (#98-10-05A) (14).

Pre-operative urine samples (at least 50 ml) were collected from 13 of the patients, and primary tumor samples from 1177 of the patients who had their tumors surgically resected at our hospital or Taipei Veterans General Hospital. Pre-operative urine samples from three patients with histological diagnosis of chronic pyelonephritis were collected as a control. All samples were immediately stored at -80°C until analysis. Clinicopathologic information for all the patients was obtained from their medical records, including demographics, clinical and surgical tumor staging, tumor size, histologic grade, the extent of disease involvement, and the status of pelvic and para-aortic lymph nodes.

LC-MS/MS analysis and database searching

Protein (100 mg) was extracted from the urine samples of two pairs of patients with UC and two pairs of patients without UC, as previously described (6, 7). Next, the protein mixtures were digested by trypsin and labeled using formaldehyde-C\textsubscript{12}H\textsubscript{2} and formaldehyde-C\textsubscript{13}D\textsubscript{2}. All samples were fractionated using liquid chromatography (NanoAcquity UltraPerformance LC; Waters, Milford, MA, USA) and analyzed using a mass spectrometer (LTQ-Orbitrap XL;
Thermo Fisher Scientific, San Jose, CA, USA). The spectra generated in both MS and CID-MS\textsuperscript{2} steps from the cleaved digest were searched using Mascot 2.3 (Matrix Science, London, UK) against the SwissProt 20110921 (532,146 sequences; 188,719,038 residues) protein databank for \textit{Homo sapiens} using a mass tolerance of ± 5 ppm for precursor ions, and ± 0.8 Da for product ions. The peptide quantification ratio (heavy/ light, UC/ non-UC) was calculated using Mascot Distiller 2.3 (Matrix Science), which uses the average peak area of the first three or four isotopic peaks across the elution profile for quantification. Manual inspections were done to exclude incorrect calculations due to low-confidence proteins (poor spectrum quality) (Supplementary Materials and Methods).

Cell culture and stable clone selection

Human embryonic kidney HEK293 and T24 grade III UC cell lines were obtained from the American Type Culture Collection and authenticated by short tandem repeat (STR) PCR profiling in 2014 and 2015 (Genomic Center, National Cheng Kung University, Taiwan). The grade II human urothelial carcinoma cell line TSGH8301 and 5637 was obtained from the Bioresource Collection and Research Center at Food Industry Research and Development Institute, Taiwan, and authenticated in 2014 by STR PCR. Both cell lines were maintained in DMEM or RPMI (Invitrogen) supplemented with 10% FBS (Hyclone) and antibiotic/antimycotic solution (Caisson Laboratories). Cells were cultured at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. The day before the experiments, cells were seeded in 6- or 10-cm dishes and starved for 24 hours. The cells were then treated with EGF (10 ng/ mL, Invitrogen) at different time
courses depending on the experiment.

TSGH8301, 5637 and HEK293 cell lines were transfected with pEGFP-N1, pEGFP-N1-SH3BGRL3, p3xflag-CMV-14 and p3xflag-CMV-14-SH3BGRL3 using Lipofectamine LTX agent (Invitrogen). T24 cell line was transfected with pcDNA6.2-GW/EmGFP-miR-neg or -SH3BGRL3 (Invitrogen). The transfectants were selected using G418 (2 mg/mL, Geneticin Selective Antibiotic (G418 Sulfate); Thermo) or blastidicin (1mg/mL; Thermo) for 6-8 weeks and verified using western blotting.

**Total RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and plasmid construction**

Cells were lysed with TRI reagent and chloroform was applied to separate the RNA at the aqueous phase. The RNA was then precipitated by isopropanol and centrifuged at 14,000 rpm for 15 minutes. Finally, the RNA was dissolved with DEPC-MQ H₂O.

Total RNA was annealed by Oligo (dT), then the following components were added in this order: M-MLV RT reaction buffer, dNTP, M-MLV reverse transcriptase, and nuclease-free water, to a final volume of 20 μL. The test tubes were mixed gently and incubated at 42°C for 1 hour and at 95°C for 5 minutes. For PCR, the templates were mixed with 10X reaction buffer, dNTPs, oligonucleotide primers, Taq polymerase and sterile MQ water. The reaction program depended on the Tm value of each pair of primers.

The plasmid construction was executed in three steps: First, the A fragment was amplified by a specific primer containing the BamHI and BglIII restriction sites. Second, PCR products
and p3xflag-CMV-14 were cut by BamHI and BglII restriction enzymes for 18 hours. Third, the two DNA fragments were ligated by DNA ligase at 4°C for 18 hours after clean-up, and then expressed by competent cells. (Supplementary Materials and Methods)

**Protein extraction, Western blotting and co-immunoprecipitation (co-IP)**

Total protein was precipitated from the patients’ urine using 20% trichloroacetic acid (Sigma-Aldrich) for 24 hours at 4°C. The protein pellet was centrifuged for 30 minutes at 14,000 rpm at 4°C, then redissolved in a solution using RIPA lysis buffer containing protease inhibitors and 1% SDS. Total protein from uroepithelial cell lines was extracted using RIPA with protease and phosphatase inhibitors, and then centrifuged for 15 minutes at 14,000 rpm at 4°C. The protein was quantified using a Lowry assay and detected using an ELISA reader at an optical density (OD) of 630 nm. For co-IP, total protein (1 mg) was incubated with primary antibody (2 μg) for 18 hours. The complex was then captured by protein G beads (Invitrogen, Carlsbad, CA, USA) for 4 hours and washed with cold PBS buffer containing protease and phosphatase inhibitors. The electrophoresis was done using a polyacrylamide gel (Ready Gel System; (Bio-Rad)). Fifty micrograms of protein was separated on a 14% SDS-PAGE at 100V for 2 hours, and it was then transferred to a 0.22-μm polyvinylidene fluoride (PVDF) membrane (Millipore) at 100 V for 90 minutes. The membrane was blocked with 5% fat-free milk for 1 hour at room temperature, and then hybridized with SH3BGRL3 (Sigma-Aldrich), E-cadherin (ab40772, Abcam), vimentin (ab92547, Abcam), EGFR (sc-31157, Santa Cruz) and beta-catenin (GTX101435, GeneTex), raf-1 (GTX107763, GeneTex), GSK-3beta (GTX111192,
GSK-3beta), at 4°C overnight. The secondary antibody (anti-rabbit, anti-mouse and anti-goat IgG-HRP; Santa Cruz Biotechnology) was used to amplify the signal as appropriate. The blots were finally developed using an enhanced chemiluminescence solution (Millipore) for 60-90 seconds.

**MTT assay**

The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. All cell lines were seeded in a 96-well plate at concentrations of 10^5 cells/well. The cells were grown in a final volume of 100 μL culture medium per well. The MTT labeling reagent (50 μL, final concentration 0.5 mg/mL) was added to each well and these were then incubated for 4 hours, after which 50 μL of dimethylsulfoxide (DMSO) was added to each well. The absorbance of the formazan product was measured at 570 nm.

**Immunohistochemistry, scoring, Immuno-fluorescent staining and confocal microscopy**

Immunohistochemical (IHC) staining, immunofluorescent staining, and confocal microscopy were done as previously described (15). Antibody for SH3BGRL3 (HPA001200, Sigma-Aldrich) at 1:500 dilution or EGFR (sc-31157, Santa Cruz Biotechnology) at 1:200 dilution was incubated overnight at 4°C. Because the intensity of immune-staining did not vary obviously, the IHC was evaluated according to the percentage of tumor cells stained (by N-H Chow) and blinded to clinical information as described previously with slight modification (4). Tissue sections showing immune-staining in less than 5% of tumor cells or lack of any
immune-reactivity were classified as negative expression. Those with a staining reaction between 5 and 25% of tumor cells were defined as low level of expression, between 25 and 50% were intermediate level of expression, and those with immune-staining in greater than 50% were defined as high level of protein expression.

Cells grown on glass coverslips were fixed with methanol/ acetone (1:1) at 4°C for 10 minutes, and permeabilized with 0.5 % Triton X-100 for 10 minutes at room temperature. To reduce the non-specific background, the cells were blocked with 5% bovine serum albumin (BSA) and washed with phosphate-buffered saline with Tween (PBST). Cells on coverslips were incubated with E-cadherin (ab40772, Abcam), vimentin (ab92547, Abcam) SH3BGRL3 (HPA001200, Sigma-Aldrich) and EGFR (sc-31157, Santa Cruz) primary antibody overnight at 4°C. After washing three times with PBST, the cells were incubated with or Alexa Fluor 594 (rabbit)-conjugated secondary antibody (Invitrogen) at room temperature for 1 hour. DAPI was used for nuclear staining. For image analysis, cells were mounted and analyzed using a confocal microscope (FV1000; Olympus) and photographed at 1800× or 2400× magnification.

Migration assays

The cell migration assay was done in 24-well trans-well plates (8.0 μm, pore size) (Millipore). The cells were seeded in the upper chamber of the trans-well system at a concentration of 2-6 × 10^4 cells/ well in 200 μL of medium, and the lower chamber was filled with 600 μL normal culture medium. After 36 hours of incubation at 37°C, 5% CO₂, the upper sides of the filters were carefully washed with PBS, and the remaining cells were removed with
a cotton swab. The cells that had migrated to the bottom side of the filter were fixed with methanol/acetone (1:1) and stained using 0.2% crystal violet. The number of migrated cells was manually counted in three random fields per filter at 6200× magnification using a phase contrast microscope.

**Statistical analysis**

All values are reported as means ± SEM (standard error of the mean). Statistical comparisons among the groups were carried out using analysis of variance (ANOVA), followed by a t test and an F test of the means of normal, benign tumor, and UC tumor tissues. Data sets that were not normally distributed were analyzed using the Mann-Whitney test for unpaired samples. Time to event distribution was estimated by means of cumulative incidence functions to take into account the patients who died (competing risk) before progression (Fine and Gray Model). Cumulative incidence curves were plotted with the STATA software program version 11 (Stata Corp., College Station, TX, USA). The mean scores of protein expression are graphically presented using error bars with 95% confidence intervals (CIs). Significance was set at $p < 0.05$. 
Results

Identification of SH3BGRL3 in the urine of patients with benign prostatic hyperplasia and UC

Urinary proteins from patients with and without UC were analyzed using LC-MS/MS. A total of 219 candidate proteins were identified (Table S1). The majority were identified by at least two unique peptides. The false discovery rate was determined to be less than 1% based on a search of the reverse protein database. Among the candidate proteins, both IgGs and serum albumin had the four highest sequence coverages (> 85%). As shown in Table S1, hemoglobin, IgGs, and serum albumin were more abundant in the pooled urine from patients with UC than from those without UC (ratio > 1). The results are consistent with prevailing notion that hematuria is a common occurrence in patients with UC, and that hemoglobin may therefore be detectable during profiling (16). The accuracy of the mass spectra results was confirmed by cytokeratin and vimentin using western blotting (Figure S1A).

Paired analysis of the urine profiling for benign prostatic hyperplasia (Table S2) and UC patients (Table S1) found that SH3BGRL3 appears in both experiments. SH3BGRL3 was identified in the current study by four unique peptides (Figure 1A, underlined) with high quality fragmentation spectra (ion score > 40), VYSTSVTGSR (Figure 1B), SQQSEVTR (Figure 1C), ATPPQIVNGDQYCGDYELFVEAVEQNTLQEFLK (Figure 1D), and IQVQLVDISQDNALR (Figure 1E), with 71% sequence coverage (Table S1). These proteins also have the highest sequence coverage in normal urine (8). Labeling (1H12C) formaldehyde and isotopic (2D13C)
formaldehyde resulted in a mass difference of 6 Da for each labeling site or a difference of 3 Da m/z for a doubly charged (z = 2) peptide, with one labeling site like VYSTSVTGSR (m/z 542.7882, 2+) (Figure 1E) derived from SH3BGRL3. As indicated, the peptide ratio was determined from each isotopic pair (Figure 1F), and the protein ratio represents the average of all ratios of peptides.

SH3BGRL3 was moderately up-regulated in the pooled urine from patients with UC compared to those without UC (average ratio: 1.4±0.1 [n = 6]). It was ranked 7th in the protein list (Table S2), signifying the potential of SH3BGRL3 as a urine marker for UC. Although SH3BGRL3 may be derived from blood incursion as a serum protein, our data are consistent with the GENT database (Gene Expression across Normal and Tumor tissue) (17), showing a higher level of SH3BGRL3 mRNA in UC than in normal tissue (Figure S1B). This study was thus designed to investigate the biological significance of SH3BGRL3 in UC.

Expression profiling of SH3BGRL3 in the urine and primary tumors of UC patients

To confirm the prediction of proteomic analysis, 13 additional urine samples from patients with UC of various histologic gradings and stages were measured for SH3BGRL3 levels. We found a positive relationship between detectable urine SH3BGRL3 and histologic grading (ρ = 0.0182) (Figures 2A and B) and muscle-invasiveness (ρ = 0.0006) (Figure 2C). A recent study reported that SH3BGRL3 is up-regulated in the secretome of human adipose tissue-derived mesenchymal stem cells upon TNF-alpha treatment (18). SH3BGRL3 was classified as a non-classical secretory protein (Figure S1C), based on analysis using the online SecretomeP 2.0
Server (19). Taking these results together, SH3BGRL3 not only may appear in the cytoplasm, but also as a secretory protein in patients' urine.

To clarify the clinical implications, expression pattern of SH3BGRL3 was analyzed on paired UC and non-tumor tissue samples (n = 32) by western blotting (Figure 2D). Up-regulated SH3BGRL3 (T > N) was detected in 71.8% of primary tumors.

A total of 55 cases of non-neoplastic uroepithelium had been constructed as an array for IHC analysis. No evidence of SH3BGRL3 or EGFR expression was found in normal uroepithelium (data not shown). IHC analysis of clinical cohort (14) demonstrated that SH3BGRL3 is expressed in 13.89% (159/1145) of bladder cancer (Table 1 and Figure 2F). SH3BGRL3 expression is significantly associated with overexpression of EGFR in primary bladder cancer (Table 1, p < 0.0001). Higher level of SH3BGRL3 expression is significantly related to muscle invasion (Figures 2G left panel, p = 0.0028). In patients with non-muscle-invasive bladder cancer (NMIBC) treated by transurethral resection without intravesical instillation (n = 179), high SH3BGRL3 expression significantly correlated with increased risk of progression (Table S3 and Figure 2H, p = 0.032).

Significance of SH3BGRL3 in cell transformation, EMT, and cell migration

Expression pattern of SH3BGRL3 was assessed in a panel of uroepithelial cell lines. SH3BGRL3 expression was positively associated with progression of urothelial carcinogenesis in vitro based on both RNA and protein levels (Figures S2A and B). Consistent with positive association of detectable urine SH3BGRL3 with histologic grading and muscle-invasiveness,
SH3BGRL3 was assumed to have a positive effect on cell transformation and migration. Survey of the Gene Expression Omnibus (GEO) database indicates that SH3BGRL3 may participate in TGF-β-induced EMT (20). To further examine these issues, transient transfection was carried out to explore the significance of SH3BGRL3 with regard to EMT phenotypes, such as E-cadherin, β-catenin, and vimentin expression (21). β-catenin was down-regulated by SH3BGRL3 overexpression, although vimentin expression remained unchanged (Figure S2C).

To elucidate the biological significance of SH3BGRL3, three stable cell lines were established from TSGH8301 cells with different levels of SH3BGRL3 expression (vector control, SH3BGRL3-Low and SH3BGRL3-High) (Figure 3A). Both SH3BGRL3 overexpressing stable cells had an increased nuclear-cytoplasmic ratio compared with vector control cells. Expression of E-cadherin and β-catenin was down-regulated (Figure 3B and D), while vimentin expression was obviously higher in SH3BGRL3 overexpressing stable cell lines (Figure 3C and D). The trans-well assay demonstrated a positive association between SH3BGRL3 expression and cell migration capacity (Figure 3F, *p <0.05 and **p <0.01).

**Growth promotion and tumorigenicity of SH3BGRL3 overexpressing stable cell lines**

Cell growth kinetics examined by MTT assay showed a positive relationship between SH3BGRL3 and cell proliferation in TSGH8301, 5637 and T24 stable cell lines (Figure 3E, *p <0.05, **p <0.01, ***p <0.001). Cyclin D1, a G1/S phase transition marker, was also up-regulated by SH3BGRL3 (Figure 3E left-upper small panel).

In terms of tumorigenicity in vivo, xenografts of the SH3BGRL3-High group were
significantly larger than those of the vector or SH3BGRL3-Low groups (Figures 3E right-upper panel and S2D, all: p < 0.02). The protein expression of SH3BGRL3 in xenografts was confirmed by western blot analysis (Figure 3E, right-upper small panel). The H & E staining of xenografts showed a poorly differentiated phenotype and more pleomorphic and bizarre nuclei in the SH3BGRL3-High group compared to the vector or SH3BGRL3-Low groups (Figure S2E).

**The interaction of SH3BGRL3 with EGFR by proline-rich residues through Grb2**

Confocal microscopy showed that SH3BGRL3 was localized near the plasma membrane, with a few signals in the nuclei of E7 immortalized urothelial cells and RT4 well-differentiated cancer cells. However, fluorescent signals were abundant in the nuclei of TSGH8301, J82, and T24 cancer cells, and the cytoplasmic signals of SH3BGRL3 were presented only in TSGH8301, J82 and T24 metastatic cancer cells (Figure S3A).

Schulze et al. (22) reported that SH3BGRL family proteins may interact with receptor tyrosine kinases (RTKs), such as EGFR and HER2, two important players in urothelial carcinogenesis (4). This prediction was supported by co-localization of SH3BGRL3 with EGFR in all five uroepithelial cell lines, irrespective of the nuclear or membranous expression patterns. Both co-IP (Figure 4A and S3E) and confocal microscopy (Figure S3B) demonstrated the interaction of SH3BGRL3 with phosphor-EGFR from 10 to 30 minutes after EGF (10 ng/mL) treatment. The interaction of SH3BGRL3 with EGFR was also demonstrated in cancer cell lines of other cell types (Figure S3D). With regard to binding site(s) on the EGFR, SH3BGRL3 binding was abolished by Y1173F mutant, while partial blockage was observed in Y1068F and
Y1086F mutants (Figures 4B and S3C). It is well known that Y1173 is related to EGFR de-phosphorylation and directly bound by SHP-1, a protein tyrosine phosphatase, and indirectly by Grb2 (23). Both Y1068 and Y1086 of EGFR were also bound by Grb2 (24, 25), and associated with the MAPK pathway.

Protein structure analysis suggested that SH3BGRL3 lacks the SH2 domain (10, 26-29) in directly binding to phosphorylated residues of EGFR. This result implies that SH3BGRL3 might indirectly interact with phosphorylated EGFR through other adaptor proteins. We thus examined the interactions of SH3BGRL3 with the SHP-1, Grb2 and PI3K p85 subunits. Co-IP demonstrated the interaction of SH3BGRL3 with Grb2 after EGF treatment (10 ng/ mL) by its proline-rich motif, but not with the SHP-1 or PI3K p85 subunits (Figure 4C). The SOS1 was also captured in SH3BGRL3 co-IP protein complex (Figure S3F). Transient transfection of SH3BGRL3 and SH3BGRL3/PP mutant (proline→alanine) in TSGH8301 cells revealed up-regulated phosphor-Akt associated with SH3BGRL3 overexpression, together with down-regulated phosphor-Erk1/2 (Figure 4D). However, this phenomenon was reversed when SH3BGRL3/ PP mutant was transfected. A cell proliferation assay further showed the growth-stimulatory effect of SH3BGRL3 in TSGH8301 cells compared to SH3BGRL3/ PP mutant (Figure 4E, all: \( p < 0.0004 \)). Endogenous SH3BGRL3 interacted with EGFR on the cell membrane, cytosol and nucleus of TSGH8301 cells after EGF treatment (Figure 4F).

**Activation of Akt-associated signaling cascade by SH3BGRL3**

In terms of signaling events associated with SH3BGRL3, up-regulated phosphor-Akt in
the SH3BGRL3 overexpressing stable cell line was demonstrated after EGF treatment (10 ng/mL), together with inhibition of GSK3-beta (Ser 9) (Figure 5A). In contrast, both phosphor-Raf-1 and phosphor-Erk1/2 were suppressed compared to vector control. Moreover, β-catenin, a downstream target of GSK3-β in the gene trans-activation (30), was trans-located to a greater extent into the nuclei of SH3BGRL3-high stable cells after EGF treatment, as compared with vector control (Figure 5B, \( p = 0.023 \)). Nuclear translocation of β-catenin was significantly inhibited by pre-treatment with LY294002 or Akt inhibitor VIII (Figure 5B, S4A and B, \( p = 1.095 \times 10^{-8} \) and \( 1.093 \times 10^{-8} \), respectively). The stimulatory effect of SH3BGRL3 on cell migration could be suppressed by pre-incubation with LY294002 and Akt inhibitor VIII (Figure 5C, \( p = 0.0019 \) and 0.0009, respectively). Cell proliferation was also inhibited by Akt inhibitor VIII (Figure 5D, \( p = 0.0059 \)). However, PD98059 had no effects on cell migration and proliferation in the SH3BGRL3-High stable cell line. Knockdown of Raf-1 (Figure S4C) inhibited cell proliferation in vector group (Figure S4D upper panel), but not in SH3BGRL3-High group (Figure S4D lower panel). Furthermore, phosphorylation of Raf-1 was down-regulated by SH3BGRL3 and was reactivated in the presence of SH3BGRL3/PP mutant (Figure S4E). These results suggest that SH3BGRL3 suppresses the Raf-1-Erk1/2 axis signaling, accompanied by activation of PI3K-Akt-related signaling cascade.
Discussion

We found that detectable urinary SH3BGRL3 is positively associated with histologic grading and muscle-invasiveness of UCs. This result seems to concur with bio-informatics analysis of protein structure (19), which predicts that SH3BGRL3 belongs to the non-classical secretory protein family (Figure S1C). A comparable expression pattern has been reported for urinary MMP-9 content (31), carcinoembryonic antigen-related cell adhesion molecule 1 (32), and fibronectin (33) in patients with UC. Therefore, measurement of urine SH3BGRL3 may become a prognostic biomarker of UC.

Tissue array analysis of clinical cohort demonstrated that SH3BGRL3 is positively associated with muscle invasion of bladder cancer and an increased risk of progression for patients with NMIBC. These results concur with the association of SH3BGRL3 expression with progression of bladder cancer predicted by Gene Expression Omnibus database in National Center for Biotechnology Information (GDS1479, Figure S1F) (34). In addition, analysis of Kaplan-Meier Plotter on-line software revealed a lower survival rate in patients with a high level of SH3BGRL3 expression in lung adenocarcinoma and breast cancer with nodal metastasis (Figures S1D and E) (35). Accordingly, evaluation of SH3BGRL3 expression status may identify a subset of bladder cancer patients who may require more intensive treatment.

In SH3BGRL3 stable cells, both spindle-shaped phenotypes and discohesiveness of cancer cells are associated with up-regulated EMT markers (suppressed E-cadherin and β-catenin with increased vimentin expression) \textit{in vitro}. The finding that aberrant cytoplasmic
expression of SH3BGRL3 appears in the sarcomatous area of bladder cancer (Figure 2F) supports for our observation \textit{in vitro}. Further analysis of the GEO database revealed that SH3BGRL3 expression is positively associated with EMT markers, e.g. TGF-β (GDS3710, Figure S1G) (20), Smad3-related pathway (GDS3985, Figure S1H) (36), TGF-β, AP1510 (GDS4361, Figure S1I) (37), and EGF (GDS2146, Figure S1J) (38). The cell cycle marker-cyclin D1 was also up-regulated by SH3BGRL3, suggesting that SH3BGRL3 promotes cell growth \textit{in vitro} and \textit{in vivo} by accelerating the G1/S phase transition. The results are consistent with our data that overexpression of SH3BGRL3 promotes the growth and mobility of UC \textit{in vitro}.

Using co-IP and confocal microscopy, SH3BGRL3 was suggested to interact with EGFR \textit{in vitro}. The observation gains strong support from the significant association of SH3BGRL3 expression with over-expression of EGFR in primary bladder cancer. EGFR has been proved to be an important prognostic factor in UC (39). It is interesting to note that both EGFR interactome (22) and protein structure of SH3BGRL3 (9) have been characterized in earlier works. However, SH3BGRL3 does not contain an SH2 domain, which is responsible for docking to phosphorylated tyrosine residues. We provided evidence that SH3BGRL3 interacts with EGFR through Grb2, an adaptor protein involved in the Raf-1-Erk1/2 axis. In addition, an inhibitory effect on the Raf-1-Erk1/2 axis, together with activation of PI3K-Akt signaling, was demonstrated in a transient transfection experiment. To clarify the importance of the proline-rich motif (10, 29) in the interactions with other proteins (40), mutation of this motif in
SH3BGRL3 blocked the interaction of SH3BGRL3 with Grb2, and inhibited cell growth. As shown in the SH3BGRL3 overexpressing stable cell line, nuclear translocation of beta-catenin, cell migration and growth rate were all suppressed by PI3K or Akt inhibitor as opposed to PD98059. These results suggest that SH3BGRL3 may be a molecular switch guiding cells to the PI3K-Akt axis after EGF treatment.

Both Ras-ERK (mitogenic) and PI3K-AKT-mTOR (survival) signaling pathways are key mechanisms for cell survival in response to extracellular cues (41). Currently, we have no rationale to explain why SH3BGRL3 activates AKT, but would inhibit ERKs in cancer cells. It is well known that cancer cells must develop stress tolerance machinery to survive under harsh conditions, such as nutrient starvation, hypoxia, and pH change in the microenvironments (42, 43). This adaptation capability contributes to malignant progression and correlates with a poor clinical outcome in several types of cancers (44). The results of current study suggest that SH3BGRL3 initiates the cross-inhibition of PI3K-AKT-mTOR signaling on Ras-Erk pathway (41), thereby driving cancer cell towards oncogene addiction. The enhanced sensitivity of SH3BGRL3 overexpressing tumor cells to gefitinib treatment in vitro support our hypothesis (Figure S4F). Nevertheless, more work is required to elucidate the mechanisms underlying SH3BGRL3 in the pathway integration of cancer cells.

Taking these findings together, SH3BGRL3 is a novel urinary biomarker for UC. Evaluation of SH3BGRL3 expression status may identify a subset of UC patients for co-targeting candidates in the design of EGFR-based cancer therapies.
Acknowledgments

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References


FIGURE LEGENDS

Figure 1. Identifying SH3BGRL3 in the urine of patients with UC.

(A) Underlining indicates the amino acid sequence of SH3BGRL3 identified by LC-MS/MS. (B) Collision-induced dissociation (CID) MS2 spectrum of VYSTSVTGSR tryptic peptide derived from SH3BGRL3. (C) CID MS2 spectrum of SQQSEVTR (m/z 481.7492, 2+) tryptic peptide derived from SH3BGRL3. (D) CID MS2 spectrum of ATPQIVNGDQYCGDYELFVEAVEQNTLQEFLK (m/z 1291.2999, 3+) tryptic peptide derived from SH3BGRL3. (E) CID MS2 spectrum of IQVQLVDISQDNALR tryptic peptide derived from SH3BGRL3. (F) The extracted isotopic pair of VYSTSVTGSR precursor ions. The peptide ratio was calculated as UC (heavy form)/non-UC (light form).

Figure 2. Clinical implications of SH3BGRL3 expression in the urine and primary tumors.

(A) Equal amounts of total protein were extracted from the urine of 13 UC patients with different stages. The relative levels of SH3BGRL3 protein were analyzed using immunoblotting of preoperatively voided urine. Detectable urinary SH3BGRL3 was associated with histological grading ($p = 0.0182$) (B) and muscle-invasiveness ($p = 0.0006$). Protein extracted from UC and non-UC tissues was assayed using immunoblotting. Expression levels of SH3BGRL3 and EGFR were normalized by beta-actin. Representative results show an upregulated SH3BGRL3 (D) and EGFR (E) in most of the primary UCs. (F) Left panel showed representative example of high level of SH3BGRL3 expression in the membrane and focal
cytoplasm of high grade urothelial carcinoma. Right panel showed aberrant strong cytoplasmic expression of SH3BGRL3 is demonstrated in the high grade sarcomatous area compared with weak staining in the low grade urothelial carcinoma element on the upper corner. (G) The quantification of IHC scoring of SH3BGRL3 in muscle-invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC). Right panel showed the quantification of IHC scoring of EGFR in muscle-invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC). (H) Progression cumulative incidence of SH3BGRL3 in UC patients. *, p<0.05; **, p<0.01; ***, p<0.001 by t test, Mann-Whitney test for unpaired samples.

Figure 3. Induction of cell transformation, EMT, cell migration and proliferation by SH3BGRL3.

(A) Hematoxylin and eosin (H&E) staining of SH3BGRL3 stable cell lines showed spindle-shaped phenotypes and discohesiveness compared with vector control group. Confocal microscopy of EMT marker expression was performed on vector and SH3BGRL3 stable cell lines. (B) Red fluorescence represents E-cadherin. The nuclei (blue fluorescence) were stained by DAPI. (C) Red fluorescence represents vimentin. (D) Immuno-blotting of EMT markers, including E-cadherin, β-catenin and vimentin, was performed on vector and SH3BGRL3 stable cell lines in TSGH8301, 5637 and T24. (E) SH3BGRL3-induced cell proliferation in TSGH8301 (left-upper panel), 5637 (left-lower panel) and T24 (right-upper panel) was assessed by MTT assay for 3-5 days. The results (means ± SD) were derived from three independent experiments. Expression of cyclin D1 in TSGH8301 stable cell lines is
shown in the small panel. SCID mice (6-8 weeks old) were subcutaneously injected with vector
or SH3BGRL3 overexpressing stable cell lines (right-lower panel). The size of tumors was
measured after 6 weeks. Expression level of SH3BGRL3 in tumors is shown in the small panel.

(F) After starving for 24 hours, TSGH8301 (upper panel), 5637 (middle panel) and T24 (lower
panel) stable cell lines (1-2 x 10^4) were seeded in the upper chamber of trans-wells and
incubated with complete culture medium for 18-24 hours. Number of migrated cells was
counted by microscopy after staining. Ten fields of migrated cells from each trans-well were
counted by microscopy. The results (means ± SD) were derived from three independent
experiments. *, p < 0.05; **, p < 0.01 by paired t test.

Figure 4. Interaction of SH3BGRL3 with EGFR through Grb2 by its proline-rich motif.

(A) HEK293-SH3BGRL3 overexpressing stable cell line was transiently transfected with EGFR
for 48 hours, starved for 24 hours and treated with EGF (10 ng/mL) at different time points.
Western blotting was performed on Co-IP using anti-EFGR antibody. Negative control (NC.)
was the lysate of HEK293-SH3BGRL3 overexpressing stable cell line, which had been starved
for 24 hours. Positive control (PC.) was the lysate of T24 cell line, which had been starved for
24 hours. (B) HEK293-SH3BGRL3 overexpressing stable cell line was transiently transfected
with wild type EGFR or different EGFR mutants for 48 hours, starved for 24 hours and treated
with EGF (10 ng/mL) for 15 minutes. Positive control (PC.) was the lysate of T24 cell line
which had been starved for 24 hours. (C) TSGH8301 was transiently transfected with p3xFlag-
SH3BGRL3, p3xFlag-SH3BGRL3/ PP mutant constructs or control vector for 24 hours, starved for 24 hours and stimulated with EGF (10 ng/ mL) for 15 minutes. Total lysate was immunoprecipitated by anti-Flag antibody and probed by Grb2, SHP-1 and PI3Kp85-beta subunit antibodies, respectively. The input samples were 5% of IP lysate. (D) TSGH8301 with wild type EGFR expression was transiently transfected with wild type SH3BGRl3 or proline-rich motif mutant, starved for 24 hours and treated with EGF (10 ng/ mL) for 5 minutes. The total lysate was detected by anti-Akt, anti-phosphor-Akt (Ser473), anti-Erk1/2 and anti-phosphor-Erk1/2 (T202 and T204), respectively. Cell treated with Akt inhibitor VIII and PD98059 were used as negative controls. The number of ratio was normalized by total protein. (E) TSGH8301 was transiently transfected with p3xFlag-SH3BGRL3, p3xFlag-SH3BGRL3/ PP mutant constructs or control vector for 24 hours. The growth kinetics was examined using MTT assay for 4 days. The results (means ± SD) were derived from three independent experiments. (F) Confocal microscopy showed that endogenous SH3BGRL3 (green fluorescence) is co-localized with EGFR (red fluorescence) in TSGH8301 cells. The results (means ± SD) were derived from three independent experiments. **, p < 0.01; ***, p < 0.001 by two-way ANOVA.

Figure 5. EGFR signaling pathway mediated by SH3BGRL3.

(A) The EGFR signaling pathway was examined in vector and SH3BGRL3 stable cell lines, respectively. All cells were starved for 24 hours, and treated with EGF (10 ng/ mL) at different time points. The phosphorylation status of signaling molecules, including EGFR, Akt, Erk1/2,
Raf-1 and GSK3-β, were examined by immuno-blotting. The ratio of phosphor-Akt and phosphor-Erk1/2 were normalized by total protein. The β-actin and Grb-2 were used as internal control. (B) Nuclear translocation of β-catenin was examined by confocal microscopy, and showed a higher proportion in SH3BGRL3 stable cell lines compared to vector control. Nuclear translocation of β-catenin was inhibited by Akt inhibitor VIII and LY294002. (C) The effect of Akt inhibitor VIII, LY294002 and PD98059 on cell migration was assessed using transwell assay. The SH3BGRL3 stable cell line was pre-incubated with inhibitors for 24 hours. Ten fields of migrated cells from each well were counted by microscopy. (D) Inhibition of cell proliferation by Akt inhibitor VIII or PD98059 was measured using MTT assay for 4 days. The SH3BGRL3 stable cell line was pre-incubated with inhibitors for 24 hours. The results (means ± SD) were derived from three independent experiments. **, p < 0.01; ***, p < 0.001 by two-way ANOVA. (E) Both Akt-and Erk-related cascades are major signaling pathways of EGFR induced by EGF. SH3BGRL3 interacts with EGFR at Y1068, Y1086 and Y1173 through Grb2 by its proline-rich motif and blocks the Erk-related cascade. Then, Akt-related cascade in cancer cell is activated for cell survival and growth. The downstream molecule-GSK3-β is phosphorylated at serine 9 with inhibition of its kinase activity, resulting in translocation of β-catenin into the nucleus.
Table 1. The association of EGFR and SH3BGRL3

<table>
<thead>
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<tr>
<td></td>
<td>Negative</td>
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<td>EGFR status</td>
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<td>Negative and Low</td>
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<tr>
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<td>986 (86.11%)</td>
<td>159 (13.89%)</td>
<td>1145 (100%)</td>
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Pearson Chi² = 103.9889  \( p < 0.0001 \)
SH3BGRL3 Protein As A Potential Prognostic Biomarker For Urothelial Carcinoma: A novel binding partner of epidermal growth factor receptor

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