SH3BGRL3 Protein as a Potential Prognostic Biomarker for Urothelial Carcinoma: A Novel Binding Partner of Epidermal Growth Factor Receptor

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Abstract

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

Experimental Design: Shot-gun proteomics using liquid chromatography-tandem mass spectrometry 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 immunoblotting of the urine (n = 13) and urothelial tumors (n = 32). Additional immunohistochemistry was performed on bladder cancer array (n = 1145) and correlated with tumor aggressiveness. Then, biologic functions and signaling pathways of SH3BGRL3 were explored using stable cell lines.

Results: The detectable urine SH3BGRL3 in patients with urothelial carcinoma was positively associated with higher histologic grading and muscle invasiveness of urothelial carcinoma. 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 nonmuscle-invasive bladder cancer (P = 0.032). SH3BGRL3 expression is significantly associated with a 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 urothelial carcinoma in vitro. SH3BGRL3 interacts with phospho-EGFR at Y1068, Y1086, and Y1173 through Grb2 by its proline-rich motif, and activates the Akt-associated signaling pathway.

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

Introduction

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

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 biologic sample in a single run of MS and distinguish protein expression levels between two biologic 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 six 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 a candidate for EGFR-based cancer therapy. Our results suggest that SH3BGRL3 is a novel cotargeting candidate in the design of EGFR-based cancer therapy.

Translational Relevance

Our prior studies indicate that EGFR, whether by itself or coexpressed with ErbB2, ErbB3, or RON, is an important predictor of tumor recurrence and survival of patients with urothelial cancer. 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 revealed that SH3BGRL3 is a novel urinary biomarker for urothelial carcinoma. SH3BGRL3 deserves investigation as a cotargeting candidate in the design of EGFR-based cancer therapy.

Materials and Methods

Patient enrollment and sample and data collection

A total of 1,190 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 (Nos. HR-97-117 and B-ER-103-214) and Taipei Veterans General Hospital (#98-10-05A; ref. 14).

Preoperative urine samples (at least 50 mL) were collected from 13 of the patients, and primary tumor samples from 1,177 of the patients who had their tumors surgically resected at our hospital or Taipei Veterans General Hospital. Preoperative urine samples from three patients with histologic 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 urothelial carcinoma and two pairs of patients without urothelial carcinoma, as previously described (6, 7). Next, the protein mixtures were digested by trypsin and labeled using formaldehyde-C12H2 and formaldehyde-C13D2. All samples were fractionated using liquid chromatography (NanoAcquity UltraPerformance LC; Waters) and analyzed using a mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific). The spectra generated in both MS and CID-MS2 steps from the cleaved digest were searched using Mascot 2.3 (Matrix Science) against the SwissProt 20110921 (532,146 sequences; 188,719,038 residues) protein databank for 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, urothelial carcinoma/non-urothelial carcinoma) 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 because of low-confidence proteins (poor spectrum quality; Supplementary Materials and Methods).

Cell culture and stable clone selection

Human embryonic kidney HEK293 and T24 grade III urothelial carcinoma cell lines were obtained from the ATCC 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 lines TSGH301 and 5637 were 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 MEM or RPMI (Invitrogen) supplemented with 10% FBS (HyClone) and antibiotics (Caisson Laboratories). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2. 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.

The TSGH301, 5637, and HEK293 cell lines were transfected with pEGFP-N1, pEGFP-N1-SH3BGRL3, p3xflag-CMV14, and p3xflag-CMV14-SH3BGR3 using Lipofectamine LTX agent (Invitrogen). The T24 cell line was transfected with
pcDNA6.2-GW/EmGF-miR-neg or pcDNA6.2-SH3BGR3L (Invitrogen). The transfecants were selected using G418 [2 mg/mL, Genetin Selective Antibiotic (G418 Sulfate); Thermo] or blasticidin (1 mg/mL, Thermo) for 6 to 8 weeks and verified using Western blotting.

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

Cells were lysed with Trizole reagent (TRI) 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 10× 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 BglII 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 resuspended in a solution using RIPA lysis buffer containing protease inhibitors and 1% SDS. Total protein from uroepithelial cell lines was extracted using RIPA with a buffer containing protease inhibitors and 1% SDS. Total protein extraction, Western blotting, and methods were done as previously described (15). Antibody for SH3BGR3L (HPA001200; Sigma-Aldrich) at 1:500 dilution or EGFR (sc-31157; Santa Cruz Biotechnology) at 1:200 dilution 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.

**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 10^3 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, immunofluorescent staining, and confocal microscopy**

Immunohistochemical (IHC) staining, immunofluorescent staining, and confocal microscopy were done as previously described (15). Antibody for SH3BGR3L (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 immunostaining 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) SH3BGR3L (HPA001200; Sigma-Aldrich) and EGFR (sc-31157; Santa Cruz Biotechnology) 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 Transwell plates (8.0 μm, pore size; Millipore). The cells were seeded in the upper chamber of the Transwell system at a concentration of 2 to 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 6,200× magnification using a phase contrast microscope.

**Statistical analysis**

All values are reported as means ± SEM. 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 urothelial carcinoma tissues. Datasets 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.). The mean scores of protein expression are graphically presented using error bars with 95% confidence intervals (CI). Significance was set at P < 0.05.

**Results**

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

Urinary proteins from patients with and without urothelial carcinoma were analyzed using LC/MS-MS. A total of 219 candidate proteins were identified (Supplementary 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 Supplementary Table S1, hemoglobin, IgGs, and serum albumin were more abundant in the pooled urine from patients with urothelial carcinoma than from those without urothelial carcinoma (ratio > 1). The results are consistent with prevailing notion that hematuria is a common occurrence in patients with urothelial carcinoma, and that hemoglobin may therefore be detectable during profiling (16). The accuracy of the mass spectra results was confirmed by cytokertin and vimentin using Western blotting (Supplementary Fig. S1A).

Paired analysis of the urine profiling for benign prostatic hyperplasia (Supplementary Table S2) and patients with urothelial carcinoma (Supplementary Table S1) found that SH3BGRL3 appears in both experiments. SH3BGRL3 was identified in this study by four unique peptides (Fig. 1A, underlined) with high-quality fragmentation spectra (ion score > 40). VYSTSVTGSR (Fig. 1B), SQSDEVTR (Fig. 1C), ATPPQQVNGDQYCCGDYELVFEAVEQNTLQELFK (Fig. 1D), and 1QVQLVDISQDNALR (Fig. 1E), with 71% sequence coverage (Supplementary Table S1). These proteins also have the highest sequence coverage in normal urine (8). Labeling (1H13C) 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+; Fig. 1E) derived from SH3BGRL3. As indicated, the peptide ratio was determined from each isotopic pair (Fig. 1F), and the protein ratio represents the average of all ratios of peptides.

SH3BGRL3 was moderately upregulated in the pooled urine from patients with urothelial carcinoma compared with those without urothelial carcinoma (average ratio: 1.4 ± 0.1 [n = 6]). It was ranked seventh in the protein list (Supplementary Table S2), signifying the potential of SH3BGRL3 as a urine marker for urothelial carcinoma. 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; ref. 17), showing a higher level of SH3BGRL3 mRNA in urothelial carcinoma than in normal tissue (Supplementary Fig. S1B). This study was thus designed to investigate the biologic significance of SH3BGRL3 in urothelial carcinoma.

Expression profiling of SH3BGRL3 in the urine and primary tumors of patients with urothelial carcinoma

To confirm the prediction of proteomic analysis, 13 additional urine samples from patients with urothelial carcinoma of various histologic gradings and stages were measured for SH3BGRL3 levels. We found a positive relationship between detectable urine SH3BGRL3 and histologic grading (P = 0.0182; Fig. 2A and B) and muscle invasiveness (P = 0.0006; Fig. 2C). A recent study reported that SH3BGRL3 is upregulated in the secretome of human adipose tissue-derived mesenchymal stem cells upon TNFα treatment (18). SH3BGRL3 was classified as a nonclassical secretory protein (Supplementary Fig. 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 urothelial carcinoma and nontumor tissue samples (n = 32) by Western blotting (Fig. 2D). Upregulated SH3BGRL3 (T > N) was detected in 71.8% of primary tumors.

A total of 55 cases of nonneoplastic 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% (159/1,145) of bladder cancer (Table 1 and Fig. 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 (Fig. 2G, left; P = 0.0028). In patients with nonmuscle-invasive bladder cancer (NMIBC) treated by transurethral resection without intravesical instillation (n = 179), high SH3BGRL3 expression significantly correlated with increased risk of progression (Supplementary Table S3; Fig. 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 (Supplementary Fig. S2A and S2B). 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 TGFβ-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 downregulated by SH3BGR1 overexpression, although vimentin expression remained unchanged (Supplementary Fig. S2C).

To elucidate the biologic significance of SH3BGR1, three stable cell lines were established from TSGH8301 cells with different levels of SH3BGR1 expression (vector control, SH3BGR1-low and SH3BGR1-high; Fig. 3A). Both SH3BGR1-overexpressing stable cells had an increased nuclear–cytoplasmic ratio compared with vector control cells. Expression of E-cadherin and β-catenin was downregulated (Fig. 3B and D), whereas vimentin expression was obviously higher in SH3BGR1-overexpressing stable cell lines (Fig. 3C and D). The Transwell assay demonstrated a positive association between SH3BGR1 expression and cell migration capacity (Fig. 3F; *, P < 0.05; **, P < 0.01).

Growth promotion and tumorigenicity of SH3BGR1-overexpressing stable cell lines

Cell growth kinetics examined by MTT assay showed a positive relationship between SH3BGR1 and cell proliferation in TSGH8301, 5637, and T24 stable cell lines (Fig. 3E; *, P < 0.05; **, P < 0.01; ****, P < 0.001). Cyclin D1, a G1–S phase transition marker, was also upregulated by SH3BGR1 (Fig. 3E left-top small panel).

In terms of tumorigenicity in vivo, xenografts of the SH3BGR1-high group were significantly larger than those of the vector or SH3BGR1-low groups (Fig. 3E right, top; Supplementary Fig. S2D, all Ps < 0.02). The protein expression of SH3BGR1 in xenografts was confirmed by Western blot analysis (Fig. 3E, right-top small panel). The hematoxylin and eosin (H&E) staining of xenografts showed a poorly differentiated phenotype and more
pleomorphic and bizarre nuclei in the SH3BGRL3-high group compared with the vector or SH3BGRL3-low groups (Supplementary Fig. 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 (Supplementary Fig. S3A).

Table 1. The association of EGFR and SH3BGRL3

<table>
<thead>
<tr>
<th>SH3BGRL3 status</th>
<th>EGFR status</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative and low</td>
<td>925 (89.55%)</td>
<td>108 (10.45%)</td>
<td>1033 (100%)</td>
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<tr>
<td>High</td>
<td>61 (54.46%)</td>
<td>51 (45.54%)</td>
<td>112 (100%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>986 (86.11%)</td>
<td>159 (13.89%)</td>
<td>1,145 (100%)</td>
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Pearson $\chi^2 = 103.9887$, $P < 0.0001$.

Schulze and colleagues (22) reported that SH3BGRL family proteins may interact with receptor tyrosine kinases (RTK), such as EGFR and HER2, two important players in urothelial carcinogenesis (4). This prediction was supported by colocalization of SH3BGRL3 with EGFR in all five uroepithelial cell lines, irrespective of the nuclear or membranous expression patterns. Both co-IP (Fig. 4A and Supplementary Fig. S3E) and confocal microscopy (Supplementary Fig. 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 (Supplementary Fig. S3D). With regard
to binding site(s) on the EGFR, SH3BGRL3 binding was abolished by Y1173F mutant, whereas partial blockage was observed in Y1068F and Y1086F mutants (Fig. 4B and Supplementary 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 (Fig. 4C). The SOS1 was also captured in SH3BGRL3 co-IP protein complex (Supplementary Fig. S3F).

Transient transfection of SH3BGRL3 and SH3BGRL3/PP mutant (proline–alanine) in TSGH8301 cells revealed upregulated phosphor-Akt associated with SH3BGR3 overexpression, together with downregulated phosphor-Erk1/2 (Fig. 4D). However, this phenomenon was reversed when SH3BGR3/PP mutant was transfected. A cell-proliferation assay further showed the growth-stimulatory effect of SH3BGR3 in TSGH8301 cells compared with SH3BGR3/PP mutant (Fig. 4E, all, \( P < 0.0004 \)). Endogenous SH3BGR3 interacted with EGFR on the cell membrane, cytosol, and nucleus of TSGH8301 cells after EGF treatment (Fig. 4F).

Activation of Akt-associated signaling cascade by SH3BGR3

In terms of signaling events associated with SH3BGR3, upregulated phosphor-Akt in the SH3BGR3-overexpressing stable cell line was demonstrated after EGF treatment (10 ng/mL), together with inhibition of GSK3-β (Fig. 5A). In contrast, both phosphor-Raf-1 and phosphor-Erk1/2 were suppressed compared with 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 SH3BGR3-high stable cells after EGF treatment, as compared with vector control (Fig. 5B, \( P = 0.023 \)). Nuclear translocation of β-catenin was
significantly inhibited by pretreatment with LY294002 or Akt inhibitor VIII (Fig. 5B and Supplementary Fig. S4A and S4B, $P = 1.095 \times 10^{-8}$ and $1.093 \times 10^{-8}$, respectively). The stimulatory effect of SH3BGRL3 on cell migration could be suppressed by preincubation with LY294002 and Akt inhibitor VIII (Fig. 5C, $P = 0.0019$ and 0.0009, respectively). Cell proliferation was also inhibited by Akt inhibitor VIII (Fig. 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 (Supplementary Fig. S4C) inhibited cell proliferation in the vector group (Supplementary Fig. S4D, top), but not in the SH3BGRL3-high group (Supplementary Fig. S4D, bottom). Furthermore, phosphorylation of Raf-1 was downregulated by SH3BGRL3 and was reactivated...
in the presence of SH3BGRL3/PP mutant (Supplementary Fig. S4E). These results suggest that SH3BGRL3 suppresses the Raf-1–Erk1/2 axis signaling, accompanied by activation of the 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 bioinformatics analysis of
protein structure (19), which predicts that SH3BGRL3 belongs to the nonclassical secretory protein family (Supplementary Fig. S1C). A comparable expression pattern has been reported for urinary MMP-9 content (31), carciomembryonic antigen-related cell adhesion molecule 1 (32), and fibronectin (33) in patients with urothelial carcinoma. Therefore, measurement of urine SH3BGRL3 may become a prognostic biomarker of urothelial carcinoma.

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 the GEO database in National Center for Biotechnology Information (GDS1479; Supplementary Fig. S1F; ref. 34). In addition, analysis of Kaplan–Meier Plotter online software revealed a lower survival rate in patients with a high level of SH3BGRL3 expression in lung adenocarcinoma and breast cancer with nodal metastasis (Fig. S1D and S1E; ref. 35). Accordingly, evaluation of SH3BGRL3 expression status may identify a subset of patients with bladder cancer who may require more intensive treatment.

In SH3BGRL3 stable cells, both spindle-shaped phenotypes and discohesiveness of cancer cells are associated with upregulated EMT markers (suppressed E-cadherin and β-catenin with increased vimentin expression) in vitro. The finding that aberrant cytoplasmic expression of SH3BGRL3 appears in the sarcomatous area of bladder cancer (Fig. 2F) supports for our observation in vitro. Further analysis of the GEO database revealed that SH3BGRL3 expression is positively associated with EMT markers, for example, TGFB (GDS3710; Supplementary Fig. S1G; ref. 20), Smad3-related pathway (GDS3985; Supplementary Fig. S1H; ref. 36), TGFβ, AP1510 (GDS4361; Supplementary Fig. S1I; ref. 37), and EGFR (GDS2146; Supplementary Fig. S1J; ref. 38). The cell-cycle marker cyclin D1 was also upregulated by SH3BGRL3, suggesting that 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 microenvironment (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 this study suggest that SH3BGRL3 initiates the cross-inhibition of PI3K–Akt–mTOR signaling on the Ras–Erk pathway (41), thereby driving cancer cell toward oncogene addiction. The enhanced sensitivity of SH3BGRL3-overexpressing tumor cells to gefitinib treatment in vitro support our hypothesis (Supplementary Fig. 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 urothelial carcinoma. Evaluation of SH3BGRL3 expression status may identify a subset of patients with urothelial carcinoma for cotargeting candidates in the design of EGFR-based cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.-Y. Chiang, N.-H. Chow


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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-Y. Chiang, C.-C. Pan, S.-H. Chen, N.-H. Chow

Writing, review, and/or revision of the manuscript: C.-Y. Chiang, H.-Y. Chang, S.-H. Chen, N.-H. Chow

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-Y. Chiang, H.-Y. Chang, M.-D. Lai, Y.-S. Tsai, P. Ling, H.-S. Liu, C.-L. Ho, S.-H. Chen, N.-H. Chow

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