Patient Mutation Directed shRNA Screen Uncovers Novel Bladder Tumor Growth Suppressors

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Abstract

Next-generation sequencing (NGS) of human bladder cancer has revealed many gene alterations compared with normal tissue, with most being predicted to be “loss of function.” However, given the high number of alterations, evaluating the functional impact of each is impractical. Here, we develop and use a high-throughput, in vivo strategy to determine which alterations are loss of function in tumor growth suppressors. Genes reported as altered by NGS in bladder cancer patients were bioinformatically processed by MutationTaster and MutationAssessor, with 283 predicted as loss of function. An shRNA lentiviral library targeting these genes was transduced into T24 cells, a nontumorigenic human bladder cancer cell line, followed by injection into mice. Tumors that arose were sequenced and the dominant shRNA constructs were found to target IQGAP1, SAMD9L, PCIF1, MED1, and KATNAL1 genes. In vitro validation experiments revealed that shRNA molecules directed at IQGAP1 showed the most profound increase in anchorage-independent growth of T24 cells. The clinical relevance of IQGAP1 as a tumor growth suppressor is supported by the finding that its expression is lower in bladder cancer compared with benign patient urothelium in multiple independent datasets. Lower IQGAP1 protein expression associated with higher tumor grade and decreased patient survival. Finally, depletion of IQGAP1 leads to increased TGFβ2 with TGFβ signaling, explaining in part how reduced IQGAP1 promotes tumor growth. These findings suggest IQGAP1 is a bladder tumor growth suppressor that works via modulating TGFβ signaling and is a potentially clinically useful biomarker.

Implications: This study used gene mutation information from patient-derived bladder tumor specimens to inform the development of a screen used to identify novel tumor growth suppressors. This included identification of the protein IQGAP1 as a potent bladder cancer growth suppressor. Mol Cancer Res; 13(9); 1–10. ©2015 AACR.

Introduction

Bladder cancer is the most common malignancy affecting the urinary system, with an expected 74 thousand new cases and 16 thousand deaths in 2015 in the United States (1). Numerous factors, including genetic polymorphisms and both genetic and epigenetic alterations, are involved in tumorigenesis, growth, and metastasis in this disease (2, 3). Recent studies reporting whole-genome or whole-exome sequencing data of bladder tumors (4–7) have enabled comprehensive cataloguing of genomic alterations. Interestingly, most mutations are computationally predicted to result in proteins with reduced expression or function.

Given that such alterations number in the hundreds, evaluating their functional impact individually is impractical. Still, evaluating their function is absolutely necessary if we are to translate sequencing data into novel avenues for therapeutic intervention. Given this need, we developed and implemented a high-throughput strategy that tests the hypothesis that some of these alterations are indeed loss of function in tumor growth suppressors. From the literature, we gathered gene alterations discovered by next-generation sequencing and identified those alterations which led to predicted loss of protein function and whose impact on tumor growth had not been previously reported in bladder cancer. We then employed RNA interference (RNAi) to reduce expression of our genes of interest and in that way, simulate loss of protein function. The effects of reduced gene expression were assessed in vivo. Therefore, plasmid-encoded short-hairpin RNAs (shRNA) were used to study in vivo xenograft phenotypes (8), where long-term target depletion is required.

An shRNA library targeting 283 candidate genes was transduced into a nontumorigenic human bladder cell line (T24), which was then injected subcutaneously into mice. Tumors that arose were sequenced to identify the shRNA responsible for promoting tumor growth. Any gene targeted by such an shRNA represents a novel tumor growth suppressor altered in human bladder cancer. Such an approach provides a novel framework for the efficient functional evaluation of hundreds of genomic alterations found in cancer and allows us to define those that are drivers rather than passengers in regulating tumor growth.
Materials and Methods

Cell lines and cell culture
Human bladder cancer cell lines T24, 253J, UMLUC3, J82, MGHU3, and HT1197 were authenticated by the University of Colorado PPSR core using an Applied Biosystems Profiler Plus Kit that analyzed 9 loci (Life Technologies 4303326). Cell ampules were resuscitated less than 2 months prior to being used in experiments in this study. Cells were cultured at 37°C with 5% CO₂ in the following media: T24: Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12) + 5% FBS, 253J and HT1197: Modified Eagle’s Medium (MEM) + 10% FBS + 0.1 mmol/L nontessential amino acids (NEAA) + 1 mmol/L sodium pyruvate, UMLUC3 and MGHU3: MEM + 10% FBS + 1 mmol/L sodium pyruvate, J82: MEM + 10% FBS + 0.1 mmol/L NEAA. All reagents for media components were obtained from Invitrogen.

Pooled shRNA library screen and lentiviral transductions
T24 cells were transduced with our Lentiviral shRNA Library comprised of MISSION Lentiplex Human shRNA constructs from Sigma Life Science and The RNAi Consortium (TRC) along with 10 μg/mL polybrene for 18 hours. Forty-eight hours after transduction, cells were selected with 2.5 μg/mL puromycin until stability was accomplished (approximately 4 days). Cells transduced with library were implanted subcutaneously into 6-week-old NCrnu/nu mice (NCI-Frederick) at 2 × 10⁶ cells/site. Mice that developed tumors were euthanized at the appropriate limits, tumors harvested, and DNA extracted using the DNAeasy Blood and Tissue Kit (Qiagen). PCR amplification from the flanking regions of the shRNA sequence was performed using the primers LPA1: TACAAAATACGTGACGTAGAAA and LPA2: TTTGTTTTTGTAATTCITTTA. PCR reactions were subjected to agarose electrophoresis, and the 350 bp bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Products were then Sanger sequenced to identify the shRNA-encoding region.

In vitro cell assays
Anchorage-independent growth in soft agar was assessed by plating 6 × 10³ cells per well in 0.5% agar in 12-well plates. Colonies were stained with Nitro-BT (Sigma) for 24 hours before imaging and counting using ImageJ. Proliferation was assessed by rehydration, 1.5 mmol/L containing a Matrigel Basement Membrane-like matrix over an 8-mm pore PET membrane (BD Biosciences). Following membrane rehydration, 1.5 × 10⁴ cells, suspended in 0.5 mL of fresh serum-free medium, were plated into the hydrated chambers and placed into wells containing 0.75 mL/well of medium plus 5% to 10% FBS and incubated at 37°C for 22 hours. The membranes were treated as in the migration assays and cells quantitated.

Invasion assays were performed using ultra low attachment round-bottom 96-well plates (Corning #7007). Cells (1 × 10⁶) were suspended in 200 μL/well and plated. After 7-day incubation at 37°C, the wells were photographed and sphere area determined in 2D using ImageJ.

qPCR
Total RNA was isolated from cells with an RNaseeasy Plus Mini kit (Qiagen). Single-stranded cDNAs were synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR reactions were then composed of cDNA, specific primers, and iQSYBR Green Supermix (Bio-Rad) and run on a q5 Real-Time PCR Detection System (Bio-Rad). Gene expression values were normalized to beta-actin controls.

Antibodies
Antibodies were obtained from the following sources: PCIF1 (Sigma; AB1407847); IQGAP1 (Life Technologies; 33-8900); SAMD9L (Sigma; HPA019461); GAPDH (Cell Signaling; 2118); pERK1/2 (Cell Signaling; 4377); ERK1/2 (Cell Signaling; 9107); α-actinin (Cell Signaling; 3134); and TGFB2 (Abcam; ab186838).

Western blot analysis and immunohistochemistry
Cell lysates were subjected to SDS-PAGE followed by transfer to polyvinylidene fluoride (PVDF) membranes (9). Membranes were blocked with 5% non-fat milk and incubated with primary antibodies overnight at 4°C. Membranes were then washed before adding the appropriate secondary antibody. Following 1-hour incubation and washing, the blots were imaged using chemiluminescence reagent (Pierce) and a ChemiDoc Imaging Station (Bio-Rad). Densitometry was performed with ImageJ.

IHC was optimized with shRNA IQGAP1-depleted and pelleted/formalin fixed human bladder cells and performed and scored (0, absent; 1+, 10%–25%; 2+, 25%–50%; 3+, >50–75%; 4+, >75% stained cells). For statistical analysis, scores were collapsed into 2 categories (0–2 and 3–4) on all tissue samples by a board-certified pathologist as described (10).

In vivo tumor study
T24 cells (2 × 10⁶) or 253J (1 × 10⁶) were implanted subcutaneously, 4 sites/mouse into 5 6-week-old NCrnu/nu mice (NCI-Frederick; tumors analyzed = 20) and measured for allograft tumor development starting 1-week after implantation and thereafter every 3 to 4 days via external measurement in two dimensions with a digital caliper to calculate tumor volumes using the equation (L × W²)/2. All animals used in this study were treated according to University of Colorado Denver and Institutional Animal Care and Use Committee (IACUC) guidelines. Animal protocols were reviewed and accepted in IACUC protocol number B-93410(12)1F.

Statistical analysis
Data were analyzed using the two-tailed Student t test with unequal variances. Error bars denote SD. Values provided are the mean ± SEM, and the differences were considered significant if P < 0.05. Kaplan–Meier curves for patients with low and high...
IQGAP1 expression groups were generated, and the corresponding log-rank P values were calculated as previously (8).

Results

In vivo functional RNAi screen identifies novel putative tumor growth suppressors

We compiled a list of genes which were reported as altered by next-generation sequencing of tumors from bladder cancer patients (4–7). This list was refined to only include those genes whose protein product was predicted to be loss of function as determined by MutationTaster (11) or MutationAssessor (12) tools. To determine if loss of function of these gene products leads to promotion of tumor growth, we first constructed an shRNA library containing 3 unique shRNA molecules targeting each of the 283 genes in our list. The use of shRNA molecules allows for the simulation of loss of protein function within a high-throughput analysis. The shRNA molecules were introduced into T24 cells as a MISSION LentiPlex Pooled shRNA library. T24 human bladder cancer cells were chosen in this study because, while they grow well in vitro, they are nontumorigenic at moderate inocula when implanted subcutaneously in immunocompromised mice (13). At 19 weeks after subcutaneous injection (Fig. 1A), we noted 14 of 20 sites had developed palpable tumors. These tumors were excised, genomic DNA extracted, and PCR amplification performed to amplify the shRNA coding region of the genomically integrated shRNA lentiviral vector. Subsequent sequencing of the PCR reaction from each tumor allowed for identification of specific shRNA molecules that were responsible for driving the growth of the tumor.

While the PCR reactions from 3 of the tumors yielded poorly defined sequence in the shRNA region, suggesting a heterogeneous pool of cells and shRNA molecules, the reactions from 11 of the tumors were unambiguous (Fig. 1B). These shRNA molecules were shown to target putative tumor suppressor genes, as their activity was responsible for promoting tumor outgrowth. The single shRNA constructs identified were targeting PXD1 C-terminal inhibiting factor 1 (PCIF1, 3 tumors), sterile alpha motif domain containing 9-like (SAMD9L, 3 tumors), IQ motif containing GTPase activating protein 1 (IQGAP1, 3 tumors), mediator complex subunit 1 (MED1, 1 tumor), and katanin p60 subunit A-like 1 (KATNAL1, 1 tumor).

To assess the relationship between these 5 genes and bladder cancer, we performed a bioinformatics analysis of all somatic mutations found in the dataset from The Cancer Genome Atlas (5). This analysis revealed, most notably, that IQGAP1 and MED1

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**Figure 1.**
RNAi screen reveals candidate tumor growth suppressors in bladder cancer. A, flow diagram of methodology for using shRNA library to identify tumor growth suppressors in vivo. B, from the 14 tumors that arose from shRNA library transduced T24 cells, 11 of them were driven by the presence of a dominant shRNA molecule that was targeting either PCIF1, SAMD9L, IQGAP1, MED1, or KATNAL1. C, analyzing the top 5 hits from the screen using Ingenuity Pathway Analysis revealed strong biologic connections between the genes IQGAP1 and MED1 and the cellular processes of tumor growth and urothelial cancer. D–F, treatment of T24 cells with shRNA molecules targeting either PCIF1, SAMD9L, or IQGAP1 leads to dramatic increases in anchorage-independent growth.

***, P < 0.001.
are biologically connected to many important genes mutated in bladder tumors, with both connected to TP53 and EP300 (Fig. 1C). In addition, many of the mutated genes in this network have previously been shown to be associated with tumor size and are found to promote human urothelial cancer growth (Fig. 1C). Thus, our novel approach appears to have identified 5 bladder cancer tumor growth suppressors.

**IQGAP1 depletion increases anchorage-independent tumor cell growth**

To begin validation of these results we assessed the ability of each shRNA, found in more than one tumor, to promote anchorage-independent growth of T24 cells in soft-agar assays. Knockdown of PCIF1, which in the screen had yielded the largest tumors at the highest rate (Fig. 1B), led to a 6-fold increase in colony formation in soft agar assays relative to control cells (Fig. 1D). Similarly, knockdown of SAMD9L led to a 5-fold increase in soft colony growth (Fig. 1E). Most dramatic was the ability of IQGAP1 knockdown to promote soft-agar colony growth (16-fold increase; Fig. 1F).

Since IQGAP1 expression correlates with tumor size in urothelial cancer and is biologically connected to many other genes altered in this disease (Fig. 1C), its loss in T24 cells exhibits the highest increase in anchorage-independent growth (Fig. 1F), we decided to focus additional studies only on IQGAP1. As a means of confirming that the changes in phenotype associated with the IQGAP1 shRNA construct used above were not the result of an off-target effect, we tested the shRNA construct #1 in another bladder cancer cell line. Knockdown of IQGAP1 in 253J bladder cancer cells also resulted in increased anchorage-independent growth (Fig. 2A). The use of a second IQGAP1 shRNA construct also led to dramatic increases in anchorage-independent growth in both 253J (Fig. 2B) and T24 (Fig. 2C) cells. In fact, knockdown of IQGAP1 was observed to increase colony formation in an additional 3 bladder cancer cell lines we tested (Fig. 2D–F). Thus, using either of two constructs to deplete cells of IQGAP1 in several bladder cancer cell lines, we conclude that loss of IQGAP1 strongly leads to increased ability of cells to grow in an anchorage-independent manner.

**Figure 2.** Depletion of IQGAP1 with multiple shRNA molecules in multiple bladder cancer cell lines consistently leads to increases in anchorage-independent growth. A, depletion of IQGAP1 with shRNA #1 in 253J bladder cancer cells led to a dramatic increase in anchorage-independent growth. B, depletion of IQGAP1 with shRNA #2 elicited the same phenotypes as shRNA #1. C, As in Fig. 1 with IQGAP1 shRNA #1, the presence of IQGP1 shRNA #2 in T24 cells leads to IQGAP1 depletion and an increase in soft-agar colonies. D–F, treatment of UMUC3, J82, and MGHU3 bladder cancer cell lines with IQGAP1 shRNA #1 leads to IQGAP1 depletion and dramatically increases anchorage-independent growth. *, P < 0.05; ***, P < 0.001.
IQGAP1 depletion enhances 253J and T24 tumor growth in vivo

We next examined the effect of IQGAP1 depletion on subcutaneous mouse xenograft growth kinetics using 253J and T24 cells, both of which have poor subcutaneous tumorigenicity and growth in immunocompromised mice (13, 14). In T24 implanted mice, the shIQGAP1 cohort reached IACUC protocol limits on tumor size at day 47 after implant. Therefore, at that time all mice in the experiment were euthanized. At this time point, the tumor volume for the shIQGAP1 cohort ranged from 42 to 358 mm³ for an average volume of 177 mm³, whereas the shCTRL cohort had 1 tumor at 91 mm³ (Fig. 3A). Although the average tumor volume appears to be only a 2-fold increase, it is important to note that T24 cells depleted of IQGAP1 had a 67% tumor take (16 tumors from 24 injections) while control cells only had a 5% incidence (1 tumor from 21 injections; Fig. 3B). Thus, loss of IQGAP1 expression was important for promoting tumor growth but even more important for promoting tumorigenicity. Western blotting of tumor lysates confirmed that IQGAP1 expression remained reduced in vivo (inset, Fig. 3A). Lysates were obtained from tumors (T1-T3) of similar size, irrespective of time points, to ensure similarity of the tumor microenvironment. The tumor lysates also revealed a reduction in phosphorylated ERK1/2. This observation is consistent with previous reports (15–17) that IQGAP1 binds ERK2 and modulates its activity due to the fact that IQGAP1 has been shown to physically interact with MEK and ERK proteins (16, 18). The decrease in tumor volume from days 30 to 40 was the result of newly measurable tumors on day 40 being added to the analysis. We also analyzed 253J cells and discovered that 253J cells depleted of IQGAP1 had an even more dramatic increase in tumorigenicity and growth compared with IQGAP1 depletion in T24 (Fig. 3C). This necessitated termination of group analysis at 32 days. At this time point, the average shIQGAP1 tumor volume was 1375 mm³ versus 33 mm³ for shCTRL (Fig. 3C). The shCTRL cohort had only a 5% tumor incidence compared with 100% in the shIQGAP1 cohort (Fig. 3D). These results provide additional validation of our screen and additional support for our hypothesis that IQGAP1 functions as a tumor suppressor in bladder tissue.

Reduced IQGAP1 level in human bladder cancer is associated with worse prognosis

To investigate if human bladder cancer has lower levels of IQGAP1 relative to normal tissue, we analyzed IQGAP1 mRNA expression in several human bladder cancer patient microarray datasets [CNU1H (19), MSKCC (20), and Stransky (21)]. This analysis revealed that IQGAP1 mRNA expression was in fact lower in tumor versus normal tissues in all three datasets (Fig. 4A). To analyze IQGAP1 protein levels in bladder cancer tumors, we optimized and then performed immunohistochemistry analysis on human bladder tumor tissue microarrays from patients that underwent radical cystectomy. Patient clinical management and follow-up have been previously described (22). Lower IQGAP1 protein levels were associated with decreased survival rates among patients who had undergone cystectomy (Fig. 4B). These data from bladder cancer patients are consistent with both our in vitro and in vivo results and strongly suggest that IQGAP1 plays a clinically relevant role as a tumor growth suppressor in bladder cancer and illustrates its potential value as a prognostic indicator.
IQGAP1 marginally affects in vitro anchorage-dependent growth, migration, and invasion

All of the analyses above reporting on the biologic impact of IQGAP1 depletion were in the context of either anchorage-independent growth or in vivo. In sharp contrast, IQGAP1 knockdown showed little effect on cells when they were analyzed in monolayer. Cells, which stably express shIQGAP1 to knockdown IQGAP1 expression (Figs. 1F and 2A), exhibited the same rate of proliferation relative to shCTRL when grown in monolayer (Fig. 5A). Second, there was no difference in the migration ability of shIQGAP1 versus shCTRL T24 cells (Fig. 5B), although IQGAP1 depletion did impact 253J migration. Third, there was no significant difference between control cells and cells depleted of IQGAP1 in invasion assays, in either T24 or 253J cell lines (Fig. 5C). Thus, in 3 different monolayer in vitro assays, we saw little to no difference in control cells compared with cells depleted of IQGAP1 in 2 bladder cancer cell lines. Consistent with this finding, we discovered that pERK1/2 levels were also unaltered in monolayer (Supplementary Fig. S1), whereas pERK1/2 levels were dramatically decreased in xenograft tumors (Fig. 4A). Finally, as another means of assessing in vitro cell growth we evaluated the impact of IQGAP1 depletion in 3-dimensional sphere growth assays performed with nonadherent plates (23). Both T24 and 253J cells readily formed tumor spheres (Fig. 5D). Furthermore, depletion of IQGAP1 in either cell line led to a significant increase in sphere volume, indicative of increased growth (Fig. 5D). Together, these experiments show that IQGAP1 depletion has by far its greatest impact when cells are grown in three dimensions, whether in vitro or in vivo.

Inhibition of TGFBR2 reverses growth phenotypes associated with IQGAP1 depletion

IQGAP1 is a major scaffold protein involved in a number of cellular processes and associates with over 90 direct or indirect binding partners (24, 25). As an initial approach to understanding the mechanisms by which loss of IQGAP1 leads to dramatic increases in tumorigenicity and tumor growth, we undertook a bioinformatics assessment to identify genes/proteins that are associated with both IQGAP1 and advanced bladder tumors. Of 909 genes identified as differentially expressed (FDR < 0.05) in a majority of 7 microarray studies of muscle invasive bladder tumors (19–21, 26–28), we identified 22 genes/proteins with a biologic connection to IQGAP1. We then used QIAGEN’s Ingenuity Pathway Analysis to look for other genes/proteins that are BIOINTEGRITY Pathway Analysis to look for other genes/proteins that are connected to these 22 genes/proteins. We discovered that half of genes/proteins were also associated with the biologic process of tumor invasion (Supplementary Fig. S2). The remaining proteins were connected to Transforming Growth Factor Beta (TGFβ) signaling, with direct and indirect associations with the growth factor TGFβ1 and its receptor TGFBR2 (Fig. 6A). These genes/proteins were also associated with the biologic process of cell viability (except CALM1), whereas all but two of them (CALM1 and CYBB) are connected to cell morphology using the IPA Knowledge Base. The probability of selecting these genes associated with cell viability and cell morphology by random chance, given all possible human genes, is 1.2 × 10−13 and

Figure 4. IQGAP1 expression suppresses tumorigenicity and mortality in bladder cancer patients. A, dot plots of IQGAP1 expression in bladder cancer patient cohorts as reported by CNUH (N = 255), MSKCC (N = 129), and Stransky (N = 62). Analyzing expression across normal and tumor samples demonstrates that normal tissues have higher IQGAP1 expression (FC, fold change). Black lines correspond to mean expression for each group. Wilcoxon Rank-Sum tests were used to generate P values and were < 0.001 in all three cohort analyses. B, Kaplan–Meier examination of IQGAP1 protein expression using tissue microarray analysis (TMA) on samples obtained from bladder cancer patients undergoing cystectomy indicates that patients grouped in the 2 lower staining scores have decreased survival after cystectomy (log-rank P value shown). Representative stained tissues are presented for IHC scores 1 and 4.
IQGAP1 Negatively Regulates Human Bladder Tumor Growth

IQGAP1 is a key regulator of cell proliferation and migration, with its loss leading to increased cell growth. This study characterizes the role of IQGAP1 in bladder tissue and identifies it as a tumor suppressor. IQGAP1 is shown to negatively regulate bladder tumor growth, with knockdown leading to increased cell proliferation and migration.

Discussion

The work presented here makes two important contributions to the field of cancer biology. First, it provides a proof of principle that mutational analysis of human tumors can inform the design of an shRNA library, which in turn can be used for the discovery of tumor suppressor genes. More broadly, this approach allows high-throughput evaluation of genomic changes in cancer to determine which are functional, thus contributing to the classification of these into driver and passenger alterations. Second, the work led to the identification of 5 novel tumor suppressor gene candidates in bladder cancer, 3 of which we validated in this study. Because the novel candidate tumor growth suppressor IQGAP1 had a bioinformatics association with another candidate, as well as an association with tumor growth and urothelial cancer, we further characterized the role of this candidate in bladder tissue. We found that depletion of IQGAP1 in 5 different bladder cancer cell lines, using 2 different shRNA molecules, led to dramatic increases in cell growth/movement in a monolayer environment relative to a 3-dimensional setting. The ability of IQGAP1 to function in bladder cancer is attributable in part to its ability to reduce TGFb signaling.

TGFb, following TGFb stimulation, which promotes SMURF1 association with TGFbR2 and subsequent ubiquitination/degradation of the receptor (30). In addition, dysregulated TGFb signaling has been shown to be pro-oncogenic (31). Therefore, we hypothesized that IQGAP1 loss results in increased TGFbR2 protein levels and subsequently increases TGFb signaling to promote cell growth. We found that depletion of IQGAP1 did in fact lead to increased TGFbR2 protein levels in bladder cancer cells (Fig. 6B). Using a reporter of TGFb signaling (3TP-Lux), we also found that shIQGAP1 cells have higher levels of TGFb-induced activity (Fig. 6C). As hypothesized, treatment of cells with TGFbR2 siRNA (Supplementary Fig. S2A) led to an abolishment of the increased TGFb signaling (Fig. 6C). To determine if these observations had biologic relevance, we performed a rescue experiment in soft-agar assays using cells transfected with TGFbR2 siRNA. Knockdown of TGFbR2 reversed the increase in soft-agar colonies induced by IQGAP1 depletion in 253J cells (Fig. 6D) and T24 cells (Supplementary Fig. S2B). This reduction in colony formation demonstrates that the ability of IQGAP1 to function as a tumor suppressor in bladder cancer is attributable in part to its ability to reduce TGFb signaling.

Loss of IQGAP1 expression has the most profound effect on cell growth when analyzed in 3-dimensional assays. A, proliferation assays revealed that loss of IQGAP1 from either T24 or 253J cells has no impact while there is a statistically significant increase in migration with loss of IQGAP1 in 253J cells. C, invasion assays reveal loss of IQGAP1 has no statistically significant effect in either T24 or 253J cells. D, when grown as tumor spheres, both T24 and 253J cells lacking IQGAP1 display a significant increase in cell growth as measured by sphere area. Representative spheres are shown in insets for each cell line.

2.8 x 10^{-13}, respectively (Fisher exact test). We also note that six of these genes (CYBB, IKBKB, IQGAP1, PTK2, SRC, and VCAM1) are associated with canonical IL8 signaling (P = 1.1 x 10^{-5}, Fisher exact test), a pathway that is associated with the epithelial–mesenchymal transition (EMT) in many types of tumors (29).

In addition, TP53, IKBKB, TGFb1, and TGFbR2 are strongly associated with EMT (P = 9.0 x 10^{-6}, Fisher exact test). A literature search revealed that IQGAP1 has been shown to bind
mutations lead to loss of function proteins. Another unique aspect of our screen was that we were only interested in identifying shRNA molecules which were present in harvested tumors, as the targets of these shRNAs represented candidate tumor growth suppressor genes. This is in contrast with determining which shRNAs were lost, as these represent proto-oncogenes, or deter-

suppressors. This is in contrast with determining which targets of these shRNAs represented candidate tumor growth suppressors. As such, these partner genes have been shown to contribute to tumorigenicity in bladder cancer (25). In addition to the screen, and subsequent validation, we found a statistically significant increase in TGFBR2 protein levels. The bar graph averages the results from 3 independent experiments. C, the use of a TGFβ reporter assay revealed that loss of IQGAP1 from 253J cells leads to enhanced TGFβ signaling. Furthermore, this enhanced signaling was attenuated with the concurrent knockdown of TGFBR2. D, knockdown of TGFBR2 expression in 253J cells results in abrogation of the previously noted increase in soft-agar colony formation that results from loss of IQGAP1.* P < 0.05; **, P < 0.01; ***, P < 0.001.

In vivo and in vivo approaches can take from a standard high-throughput, RNAi approach, which successfully yielded a number of novel tumor growth suppressors in bladder cancer.

This study was also able to determine that the ability of IQGAP1 to suppress tumorigenicity in bladder cancer cell lines is due in part to its ability to regulate TGFβ signaling. Unfortunately, the complete mechanism by which IQGAP1 acts to suppress tumorigenicity in bladder cancer is likely more complex than just having a relationship with TGFβ signaling. IQGAP1 has a large number of binding partners and, when they are dysregulated, these partners themselves have been shown to contribute to cancer (25). In addition to the screen, and subsequent in vitro and in vivo validation, we found a statistically significant correlation between IQGAP1 expression and bladder cancer patient outcomes. Such findings demonstrate a clinically relevant aspect of IQGAP1 expression and label the protein as a potential biomarker for advanced disease. Furthermore, the fact that IQGAP1 expression was found to be inversely proportional to grade suggests that IQGAP1 has an ability to inhibit tumor progression (24, 25).

Identification of a patient mutation in IQGAP1 led to the incorporation of the gene in our RNAi library. An analysis of The Cancer Genome Atlas revealed 3 additional patient missense mutations (5). This prevalence suggests that mutational status is less of a factor than decreases in gene expression. This is exactly what we observed in our study. IQGAP1 mRNA expression was lower in tumor versus normal in all 3 patient datasets we analyzed. In addition, an IHC analysis of patients following radical cystectomy revealed that lower IQGAP1 protein levels correlated less of a factor than decreases in gene expression. This is exactly what we observed in our study. IQGAP1 mRNA expression was lower in tumor versus normal in all 3 patient datasets we analyzed. In addition, an IHC analysis of patients following radical cystectomy revealed that lower IQGAP1 protein levels correlated with decreased survival. Thus, our data show that decreased expression of IQGAP1, and not necessarily mutational status, correlates strongly with negative prognosis. As a large scaffolding protein this would not be surprising as its reduced expression would affect over 90 other proteins, whereas an intact protein with missense mutations would be expected to affect fewer, assuming the protein stability and conformation were unchanged.

It is certainly worth noting that, in contrast with our findings in bladder cancer cells and patients, IQGAP1 is actually upregulated in other cancers and has been found to play a significant role in cellular functions often exaggerated in cancer (24). For example, IQGAP1 protein contributes to increased proliferation and tumorigenicity of breast epithelial cells (37). Interestingly, a similar dual role has been demonstrated between bladder and breast cancer for the protein Rho GDP Dissociation Inhibitor (GDI) Beta (RhoGD12). In bladder cancer, reduced expression of RhoGD12 is associated with decreased patient survival (38), whereas in breast cancer, higher RhoGD12 expression is associated with decreased patient survival (39). IQGAP1 protein also shows increased expression in aggressive versus less-aggressive glioma neoplasms, and has higher expression in tumor versus normal tissue of glioma, lung, and colorectal cancers (24). It has also been shown to be overexpressed in hepatocellular carcinoma and promote proliferation through Akt activation (40). With these
studies labeling IQGAP1 as an oncogene, we were surprised to discover IQGAP1 has an opposite function in bladder cancer. However, supportive of our discoveries is the finding that IQGAP1 has the ability to act as a suppressor of TGFβR2-mediated myofibroblastic activation and metastatic growth in liver (30). In addition, although IQGAP1 knockout mice have no increase in tumor incidence or progression, they do exhibit increased late-onset gastric hyperplasia (41). This, together with the inverse association of IQGAP1 levels with tumor progression, indicates that this protein may be more important in this aspect of malignancy than in tumor formation and may also have organ-specific roles in cancer progression.  

Disclosure of Potential Conflicts of Interest  
No potential conflicts of interest were disclosed.

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Conception and design: J. Hensel, D. Theodorescu  
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Molecular Cancer Research

Patient Mutation Directed shRNA Screen Uncovers Novel Bladder Tumor Growth Suppressors


Mol Cancer Res  Published OnlineFirst June 15, 2015.

Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-15-0130

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