Abstract

Purpose: The Ras family of small G proteins has been implicated in tumorigenesis, invasion, and metastasis in *in vitro* and animal model systems; however, a systematic evaluation of the state of activation, mutation, or expression of these GTPases has not been reported in any tumor type.

Experimental Design: We determined the activation state of the RalA and RalB paralogs in 10 bladder cancer cell lines with varying Ras mutation status. We sequenced RalA and RalB cDNAs from 20 bladder cancer cell lines and functionally evaluated the mutations found. We determined the expression of Ral, Ral activators, and Ral effectors on the level of mRNA or protein in human bladder cancer cell lines and tissues.

Results: We uncovered one E97Q substitution mutation of RalA in 1 of 20 cell lines tested and higher Ral activation in cells harboring mutant HRAS. We found overexpression of mRNAs for RalA and Aurora-A, a mitotic kinase that activates RalA, in bladder cancer (both *P* < 0.001), and in association with tumors of higher stage and grade. RalBP1, a canonical Ral effector, mRNA and protein was overexpressed in bladder cancer (*P* < 0.001), whereas Filamin A was underexpressed (*P* = 0.004). We determined that RalA mRNA levels correlated significantly with protein levels (*P* < 0.001) and found protein overexpression of both GTPases in homogenized invasive cancers. Available data sets suggest that RalA mRNA is also overexpressed in seminoma, glioblastoma, and carcinomas of the liver, pancreas, and prostate.

Conclusion: These findings of activation and differential expression of RalA and RalB anchor prior work in model systems to human disease and suggest therapeutic strategies targeting both GTPases in this pathway may be beneficial.

Within the Ras family of monomeric G proteins, RalA and RalB are highly similar paralogs (85% amino acid identity) that participate in diverse cellular functions. Among the processes regulated by Ral proteins are endocytosis, exocytosis, actin cytoskeletal dynamics, and transcription (reviewed in ref. 1). In some cases, Ral involvement in these processes has been shown to be mediated through effectors such as RalBP1, Sec5, Filamin A, and phospholipase D1 (Fig. 1A); in other cases, particular Ral-effector interactions responsible for downstream functions, particularly in transcription, remain to be determined. Recent results have indicated key roles for Ral proteins in tumorigenesis and metastasis (2, 3).

Ral GTPases may be activated in a Ras-dependent manner, via several guanine nucleotide exchange factors, or RalGEFs, including RalGDS (4), which is inhibited by the neurofibromatosis type 2 (NF2) gene product, Merlin (5). Activation of the Ral pathway has been recently shown to be a requirement for transformation of human cells (6, 7), and Ras-mediated transformation depends on activation of RalA (8). Based on our prior observation of Ral involvement in cancer cell motility (9), we recently reported antagonistic roles for these two GTPases, where RalB depletion by small interfering RNA or expression of activated mutant RalA both inhibit cancer cell migration (10). Another group recently found a similar role for RalB in motility through the exocyst complex in normal cells (11). Interestingly, RalA has been identified as a substrate of Aurora-A kinase, and RalA activity may be increased by phosphorylation at serine 194 (12). Finally, we recently reported a role for RalA and RalB in the transcriptional regulation of *CD24*, a metastasis-associated gene in bladder and other cancers (13) that is also a stem cell marker (14).

Given these findings, we sought to systematically investigate Ral GTPase activation state, mutation status, and expression in human bladder cancers and cell lines. Our results show that GTP-bound, activated RalA and RalB are present in cell lines...
derived from bladder cancers, and that the activation state is higher in lines harboring the G12V HRAS oncogene. We identified only one mutation, a missense mutation resulting in substitution of glutamine for glutamate at position 97 of the sequence of RalA in 1 of 20 lines screened, UM-UC-6. Most importantly, however, we identified overexpression of RalA and RalB protein in bladder cancer and overexpression of RalA mRNA in other tumors. These results support the notion that Ral GTPases are likely mediators in human bladder cancer and suggest that targeting the Ral pathway is a rational therapeutic approach.

Materials and Methods

Cell culture and gene expression profiling of bladder tumor cell lines and human bladder cancers. Forty-one human bladder cancer cells were obtained and cultured as described (15). These and primary human bladder carcinomas and normal bladder tissues were profiled on HG-U133A GeneChip arrays (Affymetrix) as described (16). The complete cohort of 65 human bladder cancers and 15 normal bladder tissues used for this study were all analyzed on the HG-U133A GeneChip Array platform and were generated by compiling our data (16) with others (17) available on National Center for Biotechnology Information Gene Expression Omnibus (18). Standardization was achieved by using original cel files for all cases, which were then processed with the dCHIP 2006 algorithm. The final human bladder tumor set includes 15 normal mucosae, 30 stage Ta, 5 T1, 6 T2, 9 T3, and 10 T4 tumors. Affymetrix probes used were RalA 214435_s_at, RalB 202100_at, RalBP1 202844_s_at, Rgl1 209568_s_at, Rgl2 209110_s_at, RalGDS 209050_s_at, Filamin A 214752_x_at, PLD1 209110_s_at, RalA 204991_s_at, Aurora-A 208079_s_at, and Merlin 204991_s_at.

Ral activation assays and site-directed mutagenesis. Ral activation was assayed and quantitated in indicated bladder cancer cell lines or transfectants using RalBP1-agarose beads from Upstate that bind activated, GTP-bound Ral GTPase (10). Percentage activation was calculated by densitometric comparison of bands of activated Ral pulled down by beads from 1,000 μg of lysate to bands of total Ral in 10 μg whole-cell lysate, divided by 100. The wild-type RalA and V23 constitutively active RalA in pcDNA3.1 expression vectors, as well as protocol for creation of the E97Q mutant RalA by site-directed mutagenesis have been described before (10). These constructs were transiently transfected into HEK293T cells (American Type Culture Collection) using the FUGENE 6 (Roche Applied Sciences) and assayed and quantitated for activation after 48 h, as reported (10).

Quantitative Ral immunoblotting of human bladder cell lines. For quantitative immunoblotting, cells from 28 different bladder cancer cell lines were grown to 80% to 90% confluence, washed with PBS, and serum-free medium was added. Cells were then incubated for 24 h, washed thrice with cold PBS, and lysed in a modified 2× SDS buffer [0.125 mol/L Tris (pH 6.8), 4% SDS, 10% glycerol] with protease inhibitors. The samples were sonicated and protein concentrations were estimated using a bicinchoninic acid protein assay kit (Pierce). DTT was added to a final concentration of 0.2 mol/L proteins were boiled, and 10 μg of proteins were loaded on gels, as reported before (10). After electroblotting to polyvinylidene difluoride membrane and blocking with 4% bovine serum albumin in TBST, RalA was detected with monoclonal anti-RalA (clone 8, BD PharMingen), as described before (9, 10, 13), whereas RalB was detected using goat polyclonal antibodies raised against RalB (R&D Systems) 1:1,000. Horseradish peroxidase–conjugated donkey anti-goat secondary antibodies (Sigma) were used for enhanced chemiluminescence detection and quantitation using an Alpha Innotech Fluorchem 8800 and AlphaEase software. Monoclonal anti–β-actin clone AC-47 (Sigma) at 0.5 μg/mL in 1% bovine serum albumin in TBST was used to detect actin expression for normalization of expression of RalA or RalB.

Ral sequencing. RalA and RalB coding sequences were examined by Transgenicom Labs. mRNA from 20 human bladder cancer cell lines (15) was extracted; cDNAs were synthesized and sequenced to determine if mutations were present in coding sequences. Two overlapping amplicons per gene were generated from cell line cDNA for downstream analysis of variants. Mutation analysis of RalA and RalB CDNA PCR products was conducted using fluorescent denaturing high-performance liquid chromatography technology and Surveyor Nuclease mismatch cleavage analysis both with the WAVE-HS System (Transgenomic). Aliquots of PCR product were scanned for variants by denaturing high-performance liquid chromatography, confirmed by Surveyor mismatch cleavage, and characterized with bidirectional sequence analysis on an ABI 3100 sequencer using BigDye V3.1 terminator chemistry (Applied Biosystems, Inc.). PCR products and sequencing reactions were cleaned for cycle sequencing and chromatography using Ampure and CleanSEQ, respectively, as per manufacturer’s standard protocols (Agencourt). For semiquantitative determination of mutant and normal allele frequencies, relative peak areas of denaturing high-performance liquid chromatography elution profiles, Surveyor mismatch cleavage products, and sequence chromatograms were examined after normalization and comparison to reference controls (using the WAVE Navigator software for denaturing high-performance liquid chromatography and Surveyor data). Sequencer software (GeneCodes) was used to visualize and align sequence chromatograms and Mutation Discovery4 and the University of California Santa Cruz genome browser5 were used for protein annotation. Primers for RalA amplicon 1 were 5′-GACTGGCTCGCGG-TGCAGATTC-3′ forward, and 5′-GTTAACATTCCACTGGCTACCTG-3′ reverse; for RalA amplicon 2 were 5′-CTACAGCTGATCTCAGGAGCAG-3′ forward and 5′-TCCCTGCCGTTCTACGAG-3′ reverse; and for RalB amplicon 1 were 5′-CTGGTCTCCCTGCTCTTTAGG-3′ forward and 5′-CCGGCTCCCTCTAGTGTCAGTT-3′ reverse; and for RalB amplicon 2 were 5′-CTGGGCACTGCTTTCCTGGGACG-3′ forward and 5′-GCGTCTCCCCCCTTTCATTG-3′ reverse. The RalA mutation was additionally independently verified by sequencing a genomic PCR product directly amplified from the UM-UC-6 genome, using primers 5′-TGACCTCAACACAGAGCAGA-3′ forward and 5′-GGATAAACACATAGTAAAGCAG-3′ reverse.

Visualization of RalA and RalB expression. Visualization of the location of the Ral A mutation identified in the RalA protein structure was carried out with INSIGHTII (Molecular Simulations, Inc.) and labeled with SHOWCASE (Silicon Graphics, Inc.).

Tumor tissue immunoblotting. Samples of adjacent normal bladder mucosa (N1,3) or muscle invasive T2, bladder tumor (T1,3) were obtained from cystectomy specimens in compliance with Human Investigation Committee of the University of Virginia and Federal guidelines. Ice-cold standard radioimmunoprecipitation assay lysis buffer plus protease and phosphatase inhibitors were used in an Omni TH tissue homogenizer (Omni) to produce protein extracts. Samples were quantitated using the bicinonic acid acid assay, and 20 μg of each was run on 4% to 20% gradient SDS-PAGE minigels (Invitrogen) as described previously (13). Tumor blots were probed for RalA or RalB (both 1:1,000, described above); RalBP1 mouse monoclonal, 1:1,000 (clone 2A1, Abnova); and Filamin A mouse monoclonal, 1:2,000 (mAb1680, Chemicon). Blots were stained with Ponceau S to ensure equal loading and transfer.

Databases and statistical analysis. The significance of expression of RalA, RalB, and associated genes with stage in bladder cancer was tested

http://www.dchip.org

http://www.mutationdiscovery.com

http://www.aacrjournals.org
using a two-tailed Student’s t test, assuming unequal variances. RalA mRNA and protein expression levels for bladder cancer cell lines were both normalized to β-actin mRNA or protein expression, respectively, and their correlation was tested by standard linear regression analysis in Statistica V6 (StatSoft). We used the differential expression function of the Oncomine10 database (19) to determine whether a statistically significant difference exists in the expression of queried genes for various comparisons.

Results

Activation state of Ral GTPases in UM-UC-6 and other human bladder cancer cell lines

Given prior reports of overexpression of epidermal growth factor receptor (20), mutations of fibroblast growth factor receptor 3 and Ras (21), and overexpression of Aurora-A kinase (22) in bladder cancer, we sought to characterize the activation state of RalA and RalB in bladder cancer cell lines. Activation assays for RalA and RalB, using the Ral binding domain of RalBP1 that specifically interacts with the GTP-bound form of RalA and RalB (10), were carried out on nine different bladder cancer cell lines as shown in Fig. 1B. Interestingly, the activation states of RalA and RalB seem to correlate with each other, particularly in cells with high levels of activation of both GTPases that harbor the G12V HRAS oncogene (Fig. 1C).

RalA but not RalB mutations are present in human bladder cancer

Cellular Ral activity may be influenced by its mutational status, including being rendered constitutively active by mutation of G23V and Q72L (Ras codons 12 and 61; ref. 10). Therefore, we evaluated the 20 human bladder cell lines for the presence of Ras mutations. We chose to sequence the cell lines because of the relative simplicity compared with tissues and as well due to the observation that only the most malignant tumors were able to generate cell lines in bladder cancer. Thus, we would expect that if present, Ras mutations would be enriched in this sample cohort, optimizing the chance for their discovery. The cell lines used for this analysis are summarized in Supplementary Table S1.

By sequencing the cDNA coding sequence of these cell lines, we found one mutation in the coding sequence of RalA from bladder cancer cell line UM-UC-6, a heterozygous G-to-C transversion at nucleotide position 589 of the RalA Refseq (NM_005402) resulting in a missense mutation of glutamate 97 to glutamine (E97Q; Fig. 2A). We confirmed this mutation by independently sequencing a PCR product containing the RalA mutation out of the genome of UM-UC-6. The crystal structure of RalA (Pdb code: 1UAD) has recently been published (23, 24). Using this structural knowledge, Fig. 2B shows a stereo view of the crystal structure of RalA, indicating the position of the E97Q mutation.

We then sought to evaluate the RalA activation state in the UM-UC-6 cell line, which harbors the mutation described above, finding an intermediate level of RalA activation between the highest, J82, and lowest, UM-UC-3, of the cell lines studied (Fig. 2C and D). Because of the relatively high level of GTP-bound E97Q mutant RalA in the UM-UC-6 cell line, we decided to directly determine whether this mutation changes the activity of the RalA protein. For this purpose, we used HEK293T cells, derived from normal embryonic kidney epithelial cells, to assay the activity of this protein in a cellular background without the complex molecular deregulation present in cancer cell lines. As shown in Fig. 2E and quantitated in Fig. 2F, this mutant, when transiently overexpressed in HEK293T cells, does not exhibit a higher level of GTP-bound activation than wild-type RalA.

Expression of Ral GTPases, their effectors, and activators in human bladder cancer

Expression of RalA, RalB, and Aurora-A mRNAs in bladder cancer. Using Affymetrix HG-U133A oligonucleotide microarray data on 65 human bladder tumors and 15 samples of normal bladder mucosa (16, 17), we evaluated the expression of Ral family GTPases as a function of transformation (cancer versus normal or invasive cancer versus normal) as well as tumor progression (invasive versus superficial cancer; Table 1; Supplementary Fig. S1). We found that RalA mRNA is significantly overexpressed in human bladder cancer (P < 0.001). Moreover, RalA overexpression is further associated with muscle invasive stage T2+ tumors compared with superficial T1a T1b (carcinoma in situ), and T2 tumors (P < 0.001; Fig. 3A). Finally, we compared RalA expression between different tumor grades, finding higher expression in grade 3 (n = 45) than grade 2 (n = 14) tumors (P < 0.001) and higher expression in grade 4 (n = 3) than grade 3 tumors (P = 0.07). Additionally, a slight overexpression of RalB mRNA in cancer was noted (1.2-fold, P = 0.03; Fig. 3B), but was not significantly associated with higher tumor grade.

Table 1 summarizes findings on association of expression of Ral pathway genes with tumor stage and grade.

Expression of activators of Ral in bladder cancer. Activation of molecules upstream of Ral that would contribute to Ral activation, including mutations of Ras (21) and both overexpression (20) or mutation (21) of receptor tyrosine kinases, have been extensively characterized in bladder cancer (reviewed in ref. 4). In vivo, activation of Ral to the GTP-bound conformation occurs through interaction with Ral-GEFs, including RalGDS, Rgl1, and Rgl2, which in turn are activated by recruitment to the membrane by their Ras Binding Domain binding to GTP-bound Ras (1). Because in vitro expression of these GEFs activates Ral (25), we evaluated the expression of these proteins in our cohort to ascertain if differential expression of these factors were associated with bladder cancer. We did not observe significant levels of overexpression of RalGDS or Rgl1 mRNA in bladder cancer or tumor progression. However, Rgl2, known commonly by the name of its mouse ortholog, Rlf, was slightly, but statistically significantly overexpressed in bladder tumors (P = 0.01, 1.4-fold).

Recent reports have identified Aurora-A kinase (12) and the NF2 gene product merlin, which inhibits RalGDS (5), as positive and negative regulators, respectively, of Ral activity. Interestingly, as reported before on the level of immunohistochemistry on bladder cancer specimens (22), Aurora-A mRNA is overexpressed in bladder tumors both when tumor tissue is compared with normal mucosa (P < 0.001) and when muscle invasive stage tumors are compared with superficial ones (P = 0.02; Fig. 3C). Moreover, as was the case with RalA,
increased Aurora-A kinase expression is associated with higher tumor grade, as we found higher levels of expression of Aurora-A in grade 3 tumors compared with grade 2 \( (P = 0.05) \) and in grade 4 tumors compared with grade 3 \( (P = 0.06; \) Table 1). Merlin mRNA expression is not significantly different between normal bladder mucosa or bladder cancer (data not shown).

Expression of Ral effectors in bladder cancer. mRNAs for the Ral effectors, RalBP1 (26), and Filamin A (27), were differentially expressed in bladder tumors compared with normal bladder tissue. Interestingly, RalBP1 was significantly overexpressed \( (P < 0.001) \), whereas Filamin A was significantly underexpressed \( (P = 0.004; \) Fig. 3D and E; Supplementary Fig. S2). However, neither was further consistently expressed with increasing tumor stage or grade. Ral has been reported to interact with EXOC2, the human homologue of the Sec5 subunit of the exocyst (28), and PLD1, phospholipase D1 (29); however, microarray probes did not reliably detect expression of these effectors above background.

RalA, but not RalB, mRNA expression correlates with protein expression

Given the results obtained above, we sought to determine if RalA protein expression is significantly associated with tumor stage in human cancers. Unfortunately, despite persistent attempts, the commercially available antibodies for RalA were not suitable for immunohistochemistry in either frozen or paraffin sections.\(^{11}\) To overcome this limitation, we reasoned that if RalA mRNA expression was proportional to RalA protein expression in bladder cancer cell lines, we could infer that human tumor expression of RalA mRNA was a reliable surrogate of the RalA protein levels in patient tumor material. Using a mouse monoclonal anti-RalA extensively published in immunoblotting (clone 8 from BD PharMingen; refs. 10, 27), we immunoblotted whole-cell extracts of 28 bladder cancer cell lines (data not shown). RalA bands were quantitated and then normalized to \( \beta \)-actin expression reprobed on the same stripped blot. Using microarray expression data for 28 human bladder cancer cell lines (15), we then normalized RalA mRNA expression to \( \beta \)-actin mRNA expression, and correlated normalized mRNA expression of RalA to normalized protein expression of RalA. As shown in Fig. 4A, we found a significant positive correlation between RalA mRNA and protein expression \( (R = 0.87, R^2 = 0.76, P < 0.001) \). This very high degree of correspondence is as good as the best found in a recent report using proteomic analysis of the NCI-60 cancer cell lines, which reported a correlation between oligonucleotide microarray expression data and protein expression level of \( R = 0.40 \) (range 0.15-0.88) across 19 gene products (30). In contrast, when we applied similar analysis to RalB expression in the same 28 cell lines, we found that there was essentially no correlation between RalB mRNA and protein expression (Fig. 4B).

Overexpression of Ral pathway proteins in bladder tumor biopsies

Because of the intriguing difference between RalA and RalB as regards correlation of mRNA and protein expression in bladder cancer cells, we assayed homogenates of tissue samples of normal bladder mucosae and muscle invasive bladder cancers taken from cystectomy specimens for their expression of these proteins by immunoblotting. Tissue homogenates were quantitated for total protein, equal amounts of protein for each sample were loaded, and blots were probed for either of these GTPases by standard means, with equivalent protein transfer validated by Ponceau S staining (not shown). As shown in Fig. 4C, invasive bladder cancers overexpress both RalA and RalB proteins. Moreover, given our findings of differential mRNA expression of RalBP1 and Filamin A in bladder cancer, we probed blots for these Ral effectors. As the mRNA results suggested, RalBP1 protein was overexpressed in four of five invasive bladder cancers, whereas Filamin A protein was underexpressed in all five (Fig. 4C, bottom).

Ral overexpression is associated with cancer and progression in other tumor types

We used Oncomine (19) to determine if differential expression of RalA and RalB mRNA exists between cancer and corresponding normal tissue in other tumors than bladder cancer. We found that Ral GTPases were overexpressed in tumor types other than bladder cancer. Table 2 shows associations between RalA overexpression and cancer in published studies of seminoma, glioblastoma, hepatocellular carcinoma, pancreatic carcinoma, and prostate cancer, and underexpression in two variants of lung cancer. Increased RalA expression is further associated with metastasis compared with primary prostate cancer \( (P < 0.001) \), a finding corroborated by another recent study, which discovered increased expression of RalA protein as a marker for metastatic prostate cancer compared with clinically localized and benign disease (31). Interestingly, this study also found that Aurora-A kinase mRNA and protein levels parallel those of RalA and are overexpressed in metastatic prostate cancer compared with localized disease. For RalB, we found reduced levels of expression in cancer, including in hepatocellular carcinoma, seminoma, ovarian carcinoma, and meningioma (Table 2).

Discussion

Recent reports have determined that activation of Ral GTPase is one of the most basic and distinct requirements for Ras-mediated transformation of human cells (6, 7) and that specific loss of function of either RalA or RalB gene product may abrogate important aspects of the cancer phenotype in different cells (9–11). However, to date, no report has systematically examined Ral GTPase expression or mutations in any single tumor type. Here, we integrate evaluation of Ral GTPase signaling, mutation, and expression data on human cancer samples to establish the clinical relevance of prior model system results.

Our survey of the activation state of 10 different bladder cancer cell lines provides both interesting clues to the activation state of Ral GTPases in human tumors as well as makes the case for more careful determination of the specificities of signaling of Ras paralogs, RalGEFs, and Ral paralogs. The highest levels of activation of RalA and RalB are exhibited in J82, T24, and a metastatic variant of T24, T24T (32, 33), all of which harbor G12V mutant HRAS, providing

\(^{11}\) Henry F. Frierson, University of Virginia Department of Pathology, personal communication.
evidence in bladder cancer cells for association of activating mutations of HRAS with higher activation states of both Ral GTPases. Conversely, RT4, which possesses wild-type HRAS (34), exhibited the lowest Ral activation levels of any cell line tested. Intriguingly, UM-UC-3, which is the only cell line studied that is known to harbor a KRAS2 mutation (21), has a low level of RalA activation and an even lower level of RalB activation, consistent with prior reports showing a lack

Fig. 1. Activation of the Ral pathway. A, schematic representation of the Ral pathway. Activated transmembrane receptor tyrosine kinases signal to Ras, resulting in Ras-GTP-dependent recruitment of RalGEFs to the membrane, where they can potentiate exchange of GDP for GTP in Ral. Then, activated Ral may signal through various known effectors and mediate diverse cellular processes (1). B, Ral activation assays were done on nine bladder cancer cell lines. Lanes 1 to 9, RT4, 5637, J82, T24, T24T, UM-UC-3, TCCSUP, HT1197, and HT1376, respectively. C, activation of Ral GTPases was quantitated (see Materials and Methods) and percentage GTP-bound RalA (white columns) and RalB (black columns) was plotted for each cell line. *, cell lines known to harbor G12V HRAS include J82, T24, and T24T; †, UM-UC-3, which harbors G12C KRAS2.
of RalA activation downstream from KRAS2 transformed cells (35).

Recent reports have focused on differential outputs of HRAS versus KRAS2 as regards canonical Ras-effector pathways (36), but, surprisingly, given the importance of the Ral pathway to transformation and tumorigenesis, the relative specificities of Ras GTPases to RalGEFs to RalA versus RalB has not been systematically determined, much less in cells derived from different tissues. Given reports of Ras, Ras-effector class, and Ral paralog–specific signaling and phenotypes, these experiments will be essential to understanding consequences of pathway output, especially as pharmaceutical methods to target specific Ras-effector pathways are developed (37). And although RalGEFs, or GEFs rendered “constitutively active” by addition of a membrane targeting “CAAX box” for lipid modification have been used extensively in in vitro experiments to activate Ral (25), their relevance to cancer in vivo has not been characterized. One recent report provides an excellent paradigm for ascertaining in vivo function of RalGEFs in tumorigenesis, by studying the effect of RalGDS knockout in an animal model of skin tumorigenesis (38). Such approaches will be necessary to determine the importance of the GEFs to tumorigenesis in vivo, and mouse models of bladder cancer, particularly driven by the activated HRAS oncogene (39), seem to be a relevant platform to test for dependency on GEF function.

We identified only one mutation in the coding sequence of RalA in the UM-UC-6 bladder cancer line, and no mutations of

![Mutation of RalA](image-url)

**Fig. 2.** Mutation of RalA. **A,** a heterozygous G-to-C transversion mutation at nucleotide position 589 of the RalA Refseq resulting in a missense mutation of glutamate 97 to glutamine. **B,** location of the E97Q mutation in the structure of RalA (Pdb code: 1UAD). Stereo view of the GNP (GTP) binding site of RalA with respect to E97. The protein is colored yellow in ribbons with backbone atoms. Magenta sticks, E97; yellow sticks, K128. Blue, GNP; red spheres, water molecules. For clarity, not all secondary structural elements or residue side chains are shown. **C,** RalA activation compared in J82, UM-UC-3, and UM-UC-6, which harbors the RalA E97Q mutation. **D,** quantitation (see Materials and Methods) of the percent GTP-bound RalA in these three cell lines from (C). **E,** activation assays were carried out on HEK293T cells transiently transfected with pcDNA3.1 expression constructs for wild-type, G23V constitutively active, or E97Q RalA. **F,** quantitation of the % GTP-bound RalA for the transfected cells in (E).
The E97Q mutation occurs in a region of the structure of this GTPase that is essential for nucleotide binding. Figure 2D shows the location of E97 with respect to the GNP binding site in the recently determined RalA crystal structure (Pdb code: 1UAD), with GNP tightly sandwiched between the residue K128, Loop 1, Turn 1, which provide extensive hydrogen bond and hydrophobic interactions to the GNP. Disturbance of K128 and any of these secondary structural elements could affect GNP binding, either positively or negatively depending on the structural perturbation. In summary, this mutation seems located in an area of RalA structure known to mediate essential functions, and the UIM-UC-6 cell line exhibits a high level of RalA activation compared with other cell lines without mutant HRAS.

Nevertheless, upon transient transfection of this mutant into normal cells, this E97Q RalA mutant behaved no differently than wild-type RalA. Although this result does not exclude that the mutant may have some gain of function in the context of a bladder cancer cell, it suggests that the intrinsic ability of RalA to bind and hydrolyze GTP is not altered by this mutation. In any case, having only found one RalA mutation in 20 bladder tumor cell lines begs the question of whether this mutation really occurs with any frequency in human cancer or whether it is but a cell culture–induced epiphenomenon. Given the findings here that RalA overexpression in bladder cancer is especially interesting because it coincides with overexpression of an activator of RalA, Aurora-A kinase (12), a phenomenon also observed in prostate cancer (31).

Aurora-A kinase is overexpressed in many tumor types, and pharmacologic inhibitors of Aurora kinase are available (40). One group recently reported that RalA (but not RalB) is a substrate for phosphorylation by Aurora-A kinase on serine 194. This phosphorylation potentiates RalA activation, anchorage-independent growth, and collagen I–induced cell migration (12). This association of overexpression of both kinase and GTPase target is intriguing, although, to our knowledge, the phosphorylation state of S194 in RalA has not been characterized in any tumor samples and will be the subject of future reports. Nevertheless, as recent reports have shown that RalA activation is essential for Ras-mediated transformation (6, 7), tumorigenesis (8), and associated with tumor maintenance in vivo (41), the potential use of Aurora kinase inhibitors to modulate RalA activity merits investigation.

We also identified overexpression of RalBP1 (26) in bladder cancer. A molecule of growing interest in cancer biology, RalBP1 has been shown to mediate survival and drug resistance (43). Although a function for RalBP1 in drug efflux has not been shown in bladder cancer cells, the potential implications of co-overexpression of this target with RalA and RalB in bladder cancer are significant. RalBP1 was discovered in a yeast two-hybrid system as a binding partner specific for active, GTP-bound Ral (44), and we are currently testing if RalBP1 has any role in potentiating RalBP1-mediated drug efflux. In

Table 1. Ral pathway genes differentially expressed in cancer and invasive cancer

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*Probe set on the Affymetrix HG-U133A oligonucleotide microarray.
†P values for expression differences tested with a two-tailed Student’s t test.
‡RalA overexpression is additionally associated with invasive stage T2-4 cancer compared with superficial T1a, T1b, and T1c cancer (P = 9.2e-5, 1.33-fold).
§Aurora overexpression is additionally associated with invasive stage T2-4 cancer compared with superficial T1a, T1b, and T1c cancer (P = 0.020, 1.33-fold).
the end, RalBP1 may prove to be an important therapeutic target in bladder cancer, as one recent report found that RalBP1 was part of a defining gene expression signature of the disease (45).

RalA has been shown to target Filamin A to induce filopodia (27). Interestingly, we found that expression of another Ral effector, Filamin A, is significantly down-regulated in bladder cancer—to our knowledge, the first reported association of loss of Filamin A expression with any tumor type. Although the significance of coincident loss of Filamin A expression and gain of RalBP1 expression in bladder tumor cells remains unknown, reports have shown that Exo84 and Sec5, two other Ral effectors, are competitive effectors of RalA function. It is intriguing to speculate that loss of one effector might potentiate signaling through another, and future experiments will determine if such a mechanism exists in bladder cancer cells.

Our finding of co-overexpression of RalA and RalB proteins in invasive bladder tumors is most interesting, however, taken in context of prior findings on Ral function. Also, the lack of correlation between mRNA and protein in RalB while a striking correlation exits between those variables in RalA is intriguing and suggests that these two paralogs may be regulated in different ways. Although a body of work has now implicated RalA in tumorigenesis of human cells, our understanding of the role of RalB in human cancer remains much more limited. We and other groups (10, 11) have discovered a role for RalB in cellular migration, one component of invasion and metastasis. Although such a model for RalA and RalB function—in tumorigenesis and

![Fig. 3. Expression of Ral pathway mRNAs.](image-url)
metastasis, respectively—is attractive, much work remains to determine the relative contributions of each to the metastatic process and if differences in effector specificity (largely unknown) explain them.

Moreover, overexpression of RalA and RalB could represent only part of a mechanism for increasing signaling through this pathway in cancer. As molecular lesioning of upstream-activating pathways of Ral is common in bladder cancer (4, 20, 21), there exists the potential that these overexpressed GTPases are additionally hyperactivated with supraphysiologic levels of GTP binding in tumors compared with normal tissue. Indeed, we observed higher levels of Ral activation in bladder cancer cell lines compared with normal HEK293T epithelial cells and particularly high levels in cell lines harboring mutant Ras. In tumors, a severalfold overexpression multiplied by severalfold increased activation state might yield a dramatic increase in total signaling output, and we propose to address this question in future reports using Ral activation assays on frozen tumor extracts.

Alternatively, recent techniques using antibody phage display have yielded recombinant antibodies that detect specifically the GTP-bound conformation of the related Rab6 small GTPase (46), and such technology could be applied to Ral.

In summary, our findings make a strong case for the pathologic relevance of both Ral GTPases, their regulators, and their effectors in human bladder cancer and other cancers and fill a current void in this area in the literature. Although our

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**Fig. 4.** Protein expression of Ral pathway genes. A, scatter plot and linear regression analysis on 28 human bladder cancer cell lines of normalized RalA mRNA level (RalA HG-U133A expression level multiplied by 10 then divided by β-actin HG-U133A expression level) against normalized RalA protein expression (RalA protein expression level divided by β-actin protein expression level from quantitative analysis of blots as described in Materials and Methods). Our microarray analysis of the cell lines has been reported before (15). B, a similar analysis to (A) done on RalB, finding a lack of correlation between normalized mRNA and protein expression. C, five tissue samples of normal bladder mucosa or muscle invasive T2+ bladder tumors (see Materials and Methods) were homogenized for protein extraction and equal protein amounts of each were Western blotted for RalA, RalB, RalBP1, and Filamin A. Blots stained with Ponceau S confirmed equivalent protein transfer (not shown). IB, immunoblot.
findings here focus on bladder cancer, the fifth most commonly diagnosed cancer in the United States (47), overexpression of RalA seems to be a common phenomenon in other tumor types as well. As such, we propose RalGTPases as targets for future therapeutic strategies.

Table 2. RalGTPase expression in other tumor types

<table>
<thead>
<tr>
<th>Probe set*</th>
<th>Type</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMAGE:260768</td>
<td>Seminoma</td>
<td>3.1e-8</td>
</tr>
<tr>
<td>39253_s_at</td>
<td>Glioblastoma</td>
<td>5.4e-5</td>
</tr>
<tr>
<td>IMAGE:230261</td>
<td>Hepatocellular carcinoma</td>
<td>7.8e-5</td>
</tr>
<tr>
<td>IMAGE:260768</td>
<td>Pancreatic adenocarcinoma</td>
<td>0.00016</td>
</tr>
<tr>
<td>IMAGE:230261</td>
<td>Prostate adenocarcinoma †</td>
<td>0.00050</td>
</tr>
<tr>
<td>IMAGE:260768</td>
<td>Lung squamous cell carcinoma</td>
<td>0.00012</td>
</tr>
<tr>
<td>IMAGE:260768</td>
<td>Lung adenocarcinoma</td>
<td>0.001</td>
</tr>
<tr>
<td>IMAGE:322617</td>
<td>Hepatocellular carcinoma</td>
<td>3.1e-8</td>
</tr>
<tr>
<td>IMAGE:322617</td>
<td>Seminoma</td>
<td>5.4e-5</td>
</tr>
<tr>
<td>IMAGE:322617</td>
<td>Ovarian adenocarcinoma</td>
<td>7.8e-5</td>
</tr>
<tr>
<td>IMAGE:260768</td>
<td>Meningioma</td>
<td>0.00016</td>
</tr>
<tr>
<td>IMAGE:322617</td>
<td>Prostate cancer</td>
<td>0.00050</td>
</tr>
</tbody>
</table>

* Probe sets used to detect RalA or RalB on various study platforms available on Oncomine.
† P value in ascending order, obtained from Oncomine for “cancer versus normal” comparison.
‡ RalA is significantly further overexpressed in metastatic prostate cancer, using the “cancer versus cancer” comparison in Oncomine to compare the “metastatic prostate cancer” with “prostate carcinoma” classes (31, 48).

References

Acknowledgments
We thank Dr. Henry Frierson, University of Virginia Department of Pathology, for his ongoing collaboration on immunohistochemistry and members of the Theodorescu Laboratory for their suggestions and assistance.


