Transcriptional Signatures of Ral GTPase Are Associated with Aggressive Clinicopathologic Characteristics in Human Cancer

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Abstract
RalA and RalB are small GTPases that support malignant development and progression in experimental models of bladder, prostate, and squamous cancer. However, demonstration of their clinical relevance in human tumors remains lacking. Here, we developed tools to evaluate Ral protein expression, activation, and transcriptional output and evaluated their association with clinicopathologic parameters in common human tumor types. To evaluate the relevance of Ral activation and transcriptional output, we correlated RalA and RalB activation with the mutational status of key human bladder cancer genes. We also identified and evaluated a transcriptional signature of genes that correlates with depletion of RalA and RalB in vivo. The Ral transcriptional signature score, but not protein expression as evaluated by immunohistochemistry, predicted disease stage, progression to muscle invasion, and survival in human bladder cancers and metastatic and stem cell phenotypes in bladder cancer models. In prostate cancer, the Ral transcriptional signature score was associated with seminal vesicle invasion, androgen-independent progression, and reduced survival. In squamous cell carcinoma, this score was decreased in cancer tissues compared with normal mucosa, validating the experimental findings that Ral acts as a tumor suppressor in this tumor type. Together, our findings show the clinical relevance of Ral in human cancer and provide a rationale for the development of Ral-directed therapies.

Introduction
Ras-like (Ral) GTPases include the homologous paralogs RalA and RalB, which have been implicated in diverse cellular functions (1). Like other small GTPases, Ral GTPases serve as a GDP/GTP conformational switch, with active signaling mediated by the GTP-loaded form, regulated by a family of Ras-dependent and -independent guanine nucleotide exchange factor (GEF) or other factors (2). A large body of literature has implicated these GTPases in key cancer phenotypes such as Ras-mediated transformation (3). This transformation is dependent specifically on RalA (4) and may be further regulated by phosphorylation (5) whereas other phenotypes such as regulation of cellular migration, invasion, and metastasis (6, 7) are attributed to either RalA or RalB, depending on the model system and cancer type evaluated.

Surprisingly, despite these important in vitro and in vivo findings, there is little evidence supporting the biologic relevance of Ral in human cancer. Unlike other GTPases, Ral mutations have not been noted in large or targeted (8) screens of common cancers. In contrast, we have observed overexpression of RalA in a small number of muscle-invasive bladder cancers (MIBC; ref. 8), whereas others noticed this in advanced prostatic adenocarcinoma (9). Neither of these studies evaluated the tumors by in situ technologies such as immunohistochemistry or in a large enough number of cases to derive sufficiently robust clinical conclusions. Furthermore, while expression of the GTPase itself contributes to the output of the Ral pathway, factors that impact GTPase activation, such as microenvironmental stimuli, posttranslational modifications including phosphorylation (5), and differential expression of downstream Ral downstream effectors, are likely to play significant roles in determining the biologic consequences of Ral expression in cancer. Ral GTPases also regulate key transcription factors such as T-cell factor (TCF), Jun, NF-κB, Stat3, HSF, E2F, the forkhead family, ZONAB, and RREB1. Targets of these pathways have been shown to include key cancer genes such as cyclin D1 (10), VEGFC (11), and CD124 (12), supportive of the important role of Ral-dependent transcription in cancers. Hence, using a transcriptional signature associated with Ral expression and/or activity may provide a useful and comprehensive picture of the Ral pathway activity in a cancer.
Here, we assess the status and clinical relevance of Ral in several human cancers by establishing and evaluating immunohistochemistry for RalA and RalB in tumor tissues. We also develop gene expression signature based on transcriptional changes induced in response to Ral depletion in cells and determine the use of this in predicting clinical outcomes in various human cancer types. Our data indicate that only the transcriptional signature of Ral is associated with human tumor characteristics and patient outcomes. In addition, this signature is also associated with experimentally proven Ral phenotypes, which validate its relevance as an accurate reporter of Ral-dependent biology. Taken together, this comprehensive approach shows for the first time the broad clinical significance of Ral in human cancer. This work also provides ample justification for the development of therapies to target the Ral pathway.

Materials and Methods

Cell lines

The BLA-40 cell line panel and its provenance has been detailed before (13). The cells have been tested/validated by gene expression profiling, supporting the bladder cancer origin of the lines, as reported in detail recently (14).

Immunohistochemistry

Immunohistochemistry (IHC) was conducted on formalin-fixed, paraffin-embedded cells and a previously described tissue microarray (TMA; ref. 15) using antibodies specific to RalA (1:1,600 dilution; Clone 8, BD Biosciences; ref. 16) and RalB (1:400 dilution; B&D Systems: ref. 5). Specificity workup used pelleted formalin-fixed, paraffin-embedded UM-UC-3 cells stably expressing FLAG vector or FLAG-tagged RalA or RalB (6) to show specificity in IHC using standard streptavidin-biotin detection (specific protocols/autostainer settings in Supplementary Information). Staining for RalA or RalB was scored semiquantitatively as either low (low-to-moderate intensity, or only focal higher expression in <50% of cells in cores examined) or high (strong, diffuse positive staining in >50% of cells in the TMA cores examined).

Ral activation assays and derivation of the Ral activation signature

Ral activation assays, which use the active, GTP-Ral-dependent interaction between the Ral-binding domain of RalBP1 conjugated to beads to selectively pull-down active RalA or RalB, which may then be quantitated and compared with total RalA or RalB by immunoblotting were as reported (4, 6). Spearman correlation between RalA and RalB percentage activation and individual probes and percentage activated RalA or RalB was conducted across microarray data generated on the BLA-40 cell line panel (13). False discovery rate was tested by random permutation testing of the Ral activation measurements for the BLA-40 cell lines, in Matlab R2010B (The MathWorks) by randomly permuting percentage activation numbers and measuring numbers of probes randomly correlated at a range of thresholds and comparing these values with those observed experimentally. The association between distributions of RalA or RalB activation and mutation status was tested by the Mann-Whitney U test, using publicly available mutation data for cell lines in the BLA-40 panel, recently tabulated by our group (14).

Derivation of the Ral transcriptional signature

We have recently reported the biologic effects of siRNA-mediated depletion of Ral GTPases in the UM-UC-3 human bladder cell line cell and profiled the transcriptional changes associated with such depletion (17). Original CEL files of these data normalized in RMA (18) as implemented in Matlab, extracting log2 expression values. After calculating fold changes for each of the HG-U133A array probes comparing Ral-intact with Ral-depleted cells, we then defined Ral transcriptional signature probes as the intersec of those altered more than 2-fold on average by depletion of both GTPases. A COXEN (COeXpression ExtrapolatioN) step was undertaken as reported (19) selecting only those candidates with concordant expression human tumors (COXEN coefficient cutoff, >0). Ral transcriptional signature scores, ranging from 0 to 1, were made from the final probe set using a correlation distance-weighted KNN prediction algorithm (WNN) that we have reported before (20). This algorithm uses group mean z-score normalized log, signature gene expression data from the cells with intact or depleted RalA and RalB as training, then uses Spearman correlation as distance metric to output a distance-weighted posterior probability for each of similarly z-score normalized clinical test cases. This probability, the Ral signature score, ranges from 0 (like siRal, signature-negative cells) to 1 (like siControl, signature-positive). These scores were designated Ral signature scores and then compared across relevant clinicopathologic groups. Cross-microarray platform comparisons were made by mapping probes by Unigene cluster ID or HUGO gene symbol, specifics for each case detailed in the Supplementary Information. Data sets used for bladder were (NCBI GEO): GSE88; GSE89; GSE19915; GSE16255; GSE31684; GSE37317; (Array Express); TABM-147; Sanchez-Carbayo and colleagues data at journal website (21); Dyrskjot and colleagues processed data (http://www.mdacc.dk/); for prostate cancer: GSE2443; GSE3803; GSE21887; GSE8702; GSE21034; GSE16560; GSE32269; GSE6956; and for squamous cell carcinoma (SCC): GSE23400; GSE2944; GSE7803.

Statistical analysis

Analysis of the association of Ral immunohistochemistry staining with clinicopathologic characteristics in bladder cancer was conducted through the \( \chi^2 \) test (Matlab) or log-rank test (Prism 5.0, GraphPad Software). For comparison of distributions of Ral transcriptional signature scores between groups of patient tumors, values were plotted and tested by the Mann-Whitney U test, Wilcoxon matched pairs test, or Kruskal-Wallis test, all in Prism 5.0, whereas association of Ral transcriptional signature scores with survival was tested with the log-rank test (Matlab).
Results

Immunohistochemical staining for RalA and RalB in human bladder cancer

Given prior observed overexpression by Western blotting and mRNA of RalA, RalB and their effectors in a small cohort of human bladder cancers (8), we were interested in examining whether Ral protein expression in human tumors was associated with clinicopathologic variables. We developed, optimized (see Supplementary Information, Figs. S1A and S1B, S2A and S2B), and then conducted immunohistochemical staining for RalA and RalB, using paralog-specific (5, 6) antibodies. We then stained and scored a TMA of archival human bladder tumor tissues that included urothelial carcinoma (n = 110) and other less common histologic variants (n = 35; ref. 15). We did not find a significant association between level of RalA or RalB staining and patient gender, pathologic stage, lymphovascular space invasion, or presence of carcinoma in situ (Supplementary Tables S1 and S2). Interestingly, tumors of non-urothelial histology (n = 35) had significantly different proportions of tumors showing low and high Ral staining compared with urothelial carcinoma (RalA, P = 0.03; RalB, P = 0.02; χ² test; Supplementary Tables S1 and S2).

In urothelial tumors (n = 110), the most common histology found in bladder cancer, we observed a trend toward decreased overall survival post-radical cystectomy in patients with intense staining for RalA (P = 0.16, log-rank; P = 0.04, Wilcoxon–Breslow, which weights early events; Fig. 1A). For RalB, there was no significant association (Fig. 1B). Stratification of survival by differing levels of RalA and RalB staining was inferior to that of RalA alone (P = 0.48; Supplementary Fig. S3).

The relationship of RalA and RalB activation to common mutations found in human bladder cancer

Activation of Ral can occur through various upstream mechanisms. However, the relationship between RalA and RalB activation status and the common pathogenetic mutations in bladder cancer remains unknown, despite the fact that a number of such lesions are known to regulate components of canonical pathways upstream of Ral. To examine this, we conducted in vitro GTPase activation assays for RalA and RalB in a large panel of human bladder cancer cell lines, the BLA-40 (13), and examined these findings with respect to cell line mutation status for 7 commonly mutated genes in bladder cancer KRAS, p53, RB, CDKN2a, PTEN, PIK3CA, and FGFR3 (14). In this large panel, activation of RalA and RalB was similarly distributed across the cell lines tested (Fig. 1C). Interestingly, RalA and RalB activations were highly correlated across the BLA-40 (r = 0.66, P < 0.0001; Fig. 1D; Supplementary Table S3). Strikingly, neither the activation of RalA nor RalB was significantly associated with mutation status for the 7 bladder cancer genes (Supplementary Table S4), suggesting independence of Ral activity from these common molecular lesions.

Development of gene expression signatures of Ral status

Current biochemical activation assays for RalA and RalB preclude testing of formalin-fixed archival bladder cancer tissues, given their requirement for rapid lysis and pull-down of activated GTPase species from fresh cells. Given that long-term clinical data are predominantly available in association with formalin-fixed tissues, we sought to overcome these limitations to evaluate such patient samples. We first used the Ral activation measurements from the BLA-40 cell lines to identify probes that correlated to Ral activation and thus generate a Ral activation signature. Unfortunately, we were unable to uncover any transcripts that correlated to RalA or RalB activation due to an unacceptably high false discovery rate (Supplementary Information and Fig. S4A and S4B).

Given these findings, we sought to develop a surrogate of Ral pathway status based on the fact that Ral GTPases alter gene expression through various transcription factors (17, 22, 23). Because tumors with the same levels of Ral protein but different levels of GTPase activation or effector interactions may induce such transcription factors to varying levels, which in turn might induce different clinical phenotypes, we hypothesized that Ral-dependent transcriptomic profiles might effectively capture pathway output that will be associated with salient clinicopathologic factors and outcomes. To directly assay for genes dependent on Ral pathway activity, by perturbing it in vivo, we decided to develop a transcriptional signature of Ral pathway status based on profiling cells depleted of RalA or RalB. We used siRNA to deplete RalA or RalB from bladder cancer cells and then profiled the resultant transcriptional changes by microarray (17). Given the significant overlap between RalA- and RalB-dependent transcriptional targets and the high degree of correlation between activation of RalA and RalB (Fig. 1D), we developed a core signature of the transcriptional program common to both RalA and RalB by choosing a union of 60 probe sets regulated by RalA and RalB depletion in human bladder cancer cells (minimum 2-fold, >100 microarray expression units difference between closest replicates; Supplementary Table S5). Importantly, and supporting the specificity of these probe sets to core Ral transcriptional signaling, we observed that this overlap between transcripts regulated by RalA and RalB was highly significant (P < 0.0001; χ² test for independence). To this set of 60 probe sets, we applied the COXEN principle (13, 19) to define a subset of 39 probesets maintaining concordant expression in a published bladder cancer microarray cohort of patients treated by radical cystectomy (n = 91; ref. 21). Importantly, as in previous COXEN implementations (19, 24), no clinical outcome or other biologic/pathologic information from the patient cohort was used in this step. Table 1 shows these probes, the genes they interrogate, their fold change in Ral intact as compared with Ral-depleted cells, and the direction of their differential expression in relevant cancer types.

Hoping to use these genes to develop a signature of the Ral pathway to examine across samples and cancer types, next, we wished to test the generality of this signature to another cell type where Ral signaling was manipulated. For this study, we used a recently published (25) data set of immortalized human embryonic kidney (HEK-HT) cells that had been stably transfected with vector control, G12V-mutant HRAS oncogene, or for G12V oncogenic Ras effector loop mutants, which interact with some specificity with the Raf pathway (G12V/T355), phosphoinositide 3-kinase (PI3K) pathway (G12V/Y40C), or RalGEF-Ral pathway (G12V/E37G).
To test the status of the Ral signature in these cell lines, we used a weighted KNN (WNN) classifier algorithm, as detailed in Supplementary Information and as reported (20). Briefly, the WNN classifier algorithm uses nonparametric (Spearman) correlation as distance metric to measure similarity of expression of Ral signature genes in a sample to be tested (e.g., one of the HEK-HT Ras samples) to the Ral-depleted or control cells, outputting a prediction score, which we call the Ral signature score, ranging from 0 to 1. This score, calculated for each outputting a prediction score, which we call the Ral signature score.

Figure 1. Expression of RalA and RalB by immunohistochemistry in 110 human urothelial bladder tumors in patients treated by radical cystectomy. A TMA of bladder carcinomas (15), stages pTa–T4, was stained with antibodies specific for RalA and RalB (see Supplementary Information). A, representative photomicrographs of strong, diffuse RalA staining (RalA high, solid) and weak RalA staining (RalA low, dashed). Kaplan–Meier analysis of overall survival in urothelial carcinoma cases (n = 110, 109 with adequate staining; Supplementary Table S1) stratified by expression level of RalA, showing a nonsignificant trend in favor of poorer overall survival for cases expressing strong RalA (P = 0.16; log-rank). Wilcoxon–Breslow testing of these curves, which weights early events, identified a significant difference (P = 0.04). B, similar microarrays to A, but for RalB, showing strong diffuse staining (blue, solid) and weak RalB staining (blue, dashed). Urothelial carcinoma cases (n = 110, 104 with adequate staining; Supplementary Table S2) were evaluated by Kaplan–Meier analysis for RalB expression, finding nonsignificant difference by log-rank or Wilcoxon–Breslow methods. C, activation of RalA and RalB. Smoothed histogram plots of RalA and RalB activation in the BLA-40 cell panel. In vitro biochemical activation assays for RalA and RalB, which use beads coated with the GTP–Ral–dependent interaction domain of the Ral effector, RalBP1 to selectively bind activated GTPase from cell lysates before detection and relative quantitation by immunoblotting, were conducted to quantitate percentage active GTP-bound RalA and RalB in matched lysates of bladder cancer cell lines (the BLA-40), RalA, red; RalB, blue. D, RalA (y-axis) and RalB (y-axis) normalized (for UM-UC-3 levels, see Supplementary Information) percentage activation (log2 scale) scatter plotted across the cell line panel, with indicated Spearman correlation. For full data, see Supplementary Table S3.

The Ral transcriptional signature characterizes invasive disease in bladder cancer

Using the 39 aforementioned Ral signature probes, we clustered the 91 tumors described above (21) with control or Ral-depleted cells and found that non–muscle-invasive (stage
pTa, pT1) tumors clustered with the Ral-depleted cells, whereas muscle invasive (stage pT2+) tumors clustered with control-treated cells (Fig. 2B). To determine quantitatively whether there is a relationship between tumor stage in this cohort and expression of the Ral signature, we again used a WNN classifier algorithm to classify the tumors based on similarity to Ral-depleted cells (like siRalA and siRalB) or control cells (like control cells, expressing Ral and its transcriptional program).

Using this approach, we observed a significant difference in distributions of Ral signature scores between non-MIBCs and MIBCs, \( P < 0.0001 \) (Fig. 2C), with non-MIBCs having lower Ral signature scores than their invasive counterparts. Importantly,

### Table 1. Probes in the Ral signature

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\(^a\)Indicated probe from the Affymetrix HG-U133A oligonucleotide microarray platform.

\(^b\)Average fold change comparing control siRNA–treated UM-UC-3 cells with cells depleted of RalA and RalB.

\(^c\)Direction of gene expression change (+ or −) in bladder carcinoma, squamous carcinoma (of esophagus, cervix, or upper aerodigestive tract), or prostatic adenocarcinoma, compared with normal respective tissue. Direction indicated if the gene were in the top 1% of genes in ≥1 study of the relevant tumor type in the Oncomine database (52).
all further implementations used the core signature (see Supplementary Information). Importantly, we applied this core signature to classify tumors of 6 additional independent cohorts of bladder tumors (26–30) profiled on 5 different microarray platforms (total additional n = 522) and found similar, significant results (Supplementary Fig. S5A–S5F).

Cells with metastatic or stem cell characteristics have high Ral transcriptional signature scores

Given the correlation of Ral signature scores with stage in patients with bladder cancer, we were interested to determine whether this score correlated with development of metastasis after surgery, especially as experimental model systems have shown an important role of RalA and RalB in mediating metastasis in vivo (5). We have recently developed a mouse model of lung metastasis using parental, poorly metastatic UM-UC-3 human bladder cancer cells. UM-UC-3 cells stably expressing firefly luciferase (Luc) for bioluminescent imaging were serially inoculated via tail vein to generate progressively more metastatic variants (LuI1 and LuI2; Fig. 3A) that were then transcriptionally profiled (31). Consistent with the importance of Ral in bladder tumor progression, we found that LuI2 had a higher Ral signature score than Luc cells (Fig. 3A). Another report used fluorescence-activated cell sorting to prospectively isolate an aggressive, highly tumorigenic/stem cell–like population of cells from SW780 bladder cancer cells, which were subsequently profiled by microarray. Suggesting a role for Ral in the stem cell phenotype, the Ral signature score was higher in highly tumorigenic/stem cell–like SW780 isolates than in parental and negative sorted populations (Fig. 3B).

Prognostic value of the Ral transcriptional signature score in human bladder cancer

Next, we examined the status of the Ral signature in tumors with respects to survival in the Sanchez-Carbayo and colleagues cohort (Fig. 3C). Using a Ral transcriptional signature score cutoff of >0.5 or <0.5 to classify as signature high or low, respectively, we found that the signature score significantly stratified cases by survival, with signature high cases showing significantly worse survival (P = 0.03; log-rank), although this difference was not independent of the association of scores with stage in multivariate Cox models (P = 0.57). Furthermore, several groups have reported that non–muscle-invasive (Ta and T1 stage) tumors that subsequently progress to muscle invasion following transurethral resection exhibit, a priori, the molecular characteristics of muscle-invasive tumors (26, 28). On the basis of these observations and our findings above showing that the Ral transcriptional signature is associated with more aggressive tumor behavior, we hypothesized that the Ral transcriptional signature might be prognostic of subsequent progression in such cases. Using 2 published microarray cohorts of non–muscle-invasive disease where progression during follow-up was documented (28, 32), we evaluated the Ral transcriptional signature score with respect to progression to muscle-invasive stage disease. We found that the score significantly stratified progression-free survival in a series (n = 29) by Dyrskjøt and colleagues (Fig. 3D, P = 0.01). Although time-to-progression data were not available...
for a second series reported by Lindgren and colleagues (n = 97, only binary, with/without progression among non–muscle-invasive cases), we again observed significantly higher Ral signature scores in cases showing progression (P = 0.04, Supplementary Fig. S5B).

Human SCC has a lower Ral transcriptional signature score than normal mucosa

Recent reports suggest that Ral may play a tumor suppressor role in SCCs (33). Hence, we reasoned that if these data have clinical significance, the Ral signature score should be lower in SCCs than in normal squamous mucosa. We evaluated the signature in a published cohort of matched SCCs and histologically normal adjacent mucosae of the esophagus (n = 53 matched tissues) evaluated by microarray (34). Strikingly, we found significantly lower Ral signature scores in SCCs than in normal mucosae (Fig. 4A, P < 0.0001). The significance of this difference over background differences in gene expression was tested by random permutation testing, observing a false discovery rate of 0.5%, supportive of the importance of the Ral transcriptional signature. The signature was then tested in a second, smaller cohort of oral SCCs (n = 26) as compared with normal mucosae (n = 12), profiled on a different microarray platform (35), finding significant difference in signature score distributions between normal and cancer (Fig. 4B, P = 0.053) consistent in direction with the first set where SCCs had lower scores than normal mucosae. To further extend these findings to an additional organ system, we tested the signature on a third cohort of SCCs (n = 21), high-grade squamous intraepithelial lesions (HSIL; n = 7), and normal mucosae of the uterine cervix (n = 10) reported by Zhai and colleagues (36). We observed the same pattern as in squamous malignancy of the esophagus and oropharynx: higher signature scores in the normal mucosae as than in the HSILs and SCCs (Fig. 4C, P = 0.01). Notably the biologically intermediate group of HSILs showed intermediate signature scores between normal and cancer.

The Ral signature in progression of prostatic adenocarcinoma

In animal models of prostate cancer, RaA and/or RaB have been associated with metastasis and androgen independence (7, 11, 37). We thus examined the status of the Ral signature with respect to important clinicopathologic surrogates of tumor aggressiveness in 2 recently published, large patient cohorts (38, 39). In patients treated by radical prostatectomy (n = 131, Taylor and colleagues; ref. 39), we did not observe significant correlations between the Ral signature scores and Gleason grade at biopsy (r = 0.11, P = 0.19) or prostatectomy (r = 0.05, P = 0.53), or with pathologic stage (P = 0.86). However, Ral signature scores could risk-stratify patients as a function of biochemical recurrence (Fig. 4D, P = 0.05). Analogous to the results described above, they showed higher Ral signature scores in these cases than in seminal vesicle invasion, a poor prognostic factor (Fig. 4E, P = 0.028).
evaluating the Ral signature score on data from the Swedish Watchful Waiting Cohort \((n = 281; \text{ref. 38})\). In this cohort, cases were incidentally diagnosed on transurethral resection (clinical T1a-b), and managed with observation only over a 10-year period. Here, Ral signature score was significantly correlated with Gleason score \((r = 0.13, P = 0.03)\) and could stratify patients by disease-specific survival \((P = 0.03, \text{Supplementary Fig. S6})\). Ral signature scores were not significantly associated with the TMPRSS-ERG fusion \((40)\) in this cohort \((P = 0.77)\). Finally, given the discrepancy noted in correlation between Ral signature and Gleason signature scores between these 2 cohorts, we analyzed one additional cohort \((n = 69)\) from the study of Wallace and colleagues \((41)\) to further test for association with Gleason grade. As in the Taylor and colleagues cohort, we observed a non-significant correlation \((r = -0.11, P = 0.37)\).

A clinically important dimension of prostate cancer biology is the issue of androgen dependence of disease. A recent report has functionally implicated RalA through induction of VEGFC upon androgen withdrawal \((11)\). To examine whether androgen withdrawal was associated with changes in the Ral signature score through long-term androgen withdrawal, as occurs during therapy, we used a published gene expression study of longitudinal (1 year) \textit{in vitro} androgen deprivation of LNCaP cells \((42)\). Comparing the Ral signature scores of replicate androgen-deprived cells with control cells over time, we observed an induction of the Ral signature scores over time \((\text{Fig. 5A}, P < 0.0001)\). Next, we examined an explanted tumor xenograft model of androgen-independent progression of prostate cancer, KCUAp-2, which has been transcriptionally profiled at baseline, at their growth nadir upon castration, and upon androgen-independent regrowth \((43)\). We found an induction of the Ral signature score over time that paralleled that observed in the LNCaP \textit{in vitro} model \((\text{Fig. 5B})\).

To determine whether such a mechanism operated in human tumors, we examined the Ral signature score in a data set of microarray profiled, microdissected androgen-dependent \((n = 10)\), and androgen-independent \((n = 10)\) prostate tumors \((44)\). We observed that the Ral signature score distributions differed significantly, with higher scores in androgen-independent disease \((\text{Fig. 5C}, P = 0.005)\). Random permutation testing suggested that the observed degree of difference between androgen-dependent and
observed again a highly significant metastatic cases (N = 10) than in androgen-dependent (n = 10) cases (Mann–Whitney test, signature scores plotted, and medians indicated per group, black lines). D, a similar higher Ral signature score in androgen-independent cases in a second cohort (ref. 45; n = 8) than in androgen-dependent cases (n = 18). E, similar higher Ral signature score in androgen-independent metastatic tissues of a third cohort (ref. 46; n = 29) than in androgen-naive primary cases (n = 22).

Discussion

RalA and RalB have been implicated in transformation, regulation of survival, migration, and metastasis. However, although ample dysregulation of pathways upstream of Ral signaling, including mutation of Ras paralogs, has been described in human tumors, mutations of RalA and RalB have not been found. In addition, despite observations of differential expression or activation of RalA or RalB in small cohorts of bladder (8), prostate (9), squamous (33), and pancreatic cancers, there is no evidence supporting the relevance of this pathway on clinical outcome in large patient series that include multiple cancer types.

To our knowledge, our findings provide the first evidence supporting a role of Ral in mediating clinically meaningful phenotypes in human cancer. First, we showed that a Ral signature, derived from comparing bladder cancer cells with and without depletion of RalA and RalB (loss of function) was associated, in the proper, opposite direction, with Ras mutant activity, including specifically the E37G Ras effector loop mutant stimulating RalGEF pathway, (both gain of function) in another cell type (human embryonic kidney cells; ref. 25). These findings argue strongly for the generality of this signature and support our downstream studies comparing the signature in different tissue types. Also, findings of our novel Ral transcriptional signature closely parallel experimentally shown roles of Ral in model systems. This is perhaps most striking for SCCs, where Ral was shown to act as a tumor suppressor in experimental systems, in contrast to its role in other models (33). This relevance of this role in human cancer was supported by our findings using the Ral transcriptional signature score, which was lower in tumors compared with normal mucosa. This finding also speaks of the specificity of the Ral signature to Ral biology. For example, if the score were simply a surrogate of a global phenotype such as transformation, we would not expect lower signature scores in SCCs than in normal mucosa.

One key aspect of Ral biology is the ability of RalA and RalB to regulate transcription through a number of transcriptional pathways including TCF, Jun, NF-κB, Stat3, HSF, and forkhead family members (2). We recently added the metastasis and stem cell–associated gene, CD24, to this list (12, 31), and have also implicated the RREB1 transcription factor pathway therein (17, 23). However, it bears consideration that a number of the Ral signature genes (Table 1) have been shown to play important roles in bladder and other cancers. Clusterin has substantial literature in bladder cancer as an antiapoptotic protein, both as a biomarker for disease aggression and, when inhibited, as a chemosensitizing agent to the key chemotherapeutic drug, gemcitabine; in prostate cancer, an inhibitor of clusterin, catusirsen (OGX-011) has shown promise as a chemosensitizing agent in clinical trials castration-resistant disease (47). Overexpression of replication protein A1 (RPA1), which we found to be lower in Ral signature–positive cells, has been recently shown to be a positive prognostic factor in bladder cancer (48), which correlates our signature findings. The L1CAM adhesion molecule, higher in signature-positive cells, has an extensive literature in invasion and metastasis (49) and may be involved in

Figure 5. The Ral transcriptional signature score is associated with androgen status in prostate cancer. A, using published expression profiling data for LNCaP cells treated with control or charcoal-stripped (steroid hormone-free) medium over a time course of 12 months (42), we observed significant and durable induction of the Ral transcription over time in androgen-deprived (charcoal-stripped serum (CSS), grey/upper line) as compared with control full serum-treated cells (full serum, black/lower line), Mann–Whitney test. B, quadruplicate KuCaP-2 (43) xenografts were analyzed at androgen-dependent baseline (AD), at castration-induced growth nadir (Tx), and during androgen-independent (AI) regrowth. A significantly higher Ral signature score was seen in treated and androgen-independent tumors (Mann–Whitney test of all treated vs. baseline replicates, plot shows median plus 95% confidence interval [CI]), consistent with the in vitro and in vivo results in A and B, we observed significantly higher Ral signature scores in a published cohort (44) of androgen-independent tumors (n = 10) than in androgen-dependent (n = 10) cases (Mann–Whitney test, signature scores plotted, and medians indicated per group, black lines). C, a similar higher Ral signature score in androgen-independent cases in a second cohort (ref. 45; n = 8) than in androgen-dependent cases (n = 18). E, similar higher Ral signature score in androgen-independent metastatic tissues of a third cohort (ref. 46; n = 29) than in androgen-naive primary cases (n = 22).

-independent cases was specific to the Ral transcriptional signature rather than global differences in transcription (false discovery rate, 1%). We further tested this approach on a second cohort (45) of androgen-dependent (n = 18) and androgen-independent cases (n = 18), profiled on a different, custom microarray platform (Fig. 5D, P = 0.02), thus generalizing these observations. For further validation of the Ral signature in the setting of androgen-independent metastatic prostate cancer, we used an additional data set, reported by Stanbrough and colleagues (46), where primary, hormone-naive tissues (n = 22) and androgen-independent bone metastatic cases (N = 29) were profiled by microarray. We observed again a highly significant difference in Ral signature scores, with increased scores in androgen-independent cases (Fig. 5E, P < 0.0001), similar to the 2 other cohorts. Again, as in the Swedish Watchful Waiting cohort above, Ral signature scores were not associated with the TPRRSS-ERG fusion status of the cases (P = 0.32).
cooperative signaling with the aforementioned CD24. Taken together these and prior findings suggest that Ral may coordinately regulate genes involved in aggression and metastasis.

Interestingly, on the basis of this foundation and observations in the manuscript on a cohort of bladder cancer cell isolates stratified for their stem cell–like properties (50), we implicate for the first time Ral biology with this key phenotype. This is particularly interesting given the finding that human pancreatic cancer stem cells express high levels of CD24 (51) and pancreatic cancer is a K-Ras/Ral-driven cancer (4). Because Ral regulates CD24 (12), the data presented here implicating Ral in the cancer stem cell phenotype are consistent with the literature. These findings suggest that Ral might have a role in regulating this key cellular subpopulation thought to play a therapeutically central role in patients. Hence, targeting the Ral GTPase would help delete this population of cells, reducing drug resistance with consequently beneficial clinical results.

The core signature of Ral-dependent transcription shared by RaLa and RaBb is a pervasive feature of MBCs, all the more striking given its consistency across a large number of cohorts from different institutions, geographical locations, and profiled on different microarray platforms. In the case of one cohort by Sanchez-Carbayo and colleagues where survival data were available, we found that the signature was associated with survival, consistent with the role of Ral in experimental metastasis (5) as well as our observation herein that the Ral signature is associated with metastatic competence in experimental models.

In prostate cancer, reports have shown roles for Ral in model phenotypes of progression (7), including metastasis to bone (37) and induction of VEGF under androgen ablation (11). Here, we found significant association of the Ral signature with emergence of androgen independence in vitro and in vivo in experimental models and in 2 patient cohorts. These findings implicate Ral in the very center of perhaps the most clinically important aspect of prostate cancer management, namely, recurrence under androgen ablation therapy, a key driver of mortality in this disease. Targeting the Ral pathway in simultaneous combination with androgen deprivation might reduce the emergence of the hormone-refractory state and deserves investigation as a therapeutic strategy. Importantly, excepting in the Swedish Watchful Waiting cohort of incidentally diagnosed [i.e., not prostate-specific antigen (PSA)-screened, as modern cohorts are low-stage disease], we found no correlation between the Ral signature score and Gleason grade. While this exception identifies another molecular difference between the Swedish cohort and modern PSA-screened biopsy populations (such as that have been seen before regarding prevalence of ERG rearrangements), overall the Ral signature was uncorrelated to Gleason grade, suggesting that it could be adapted to provide independent or complementary prognostic data.

In summary, these findings provide the first conclusive evidence from human tumors that Ral GTPase status is clinically important. Furthermore, they provide a new tool to the scientific community, the Ral transcriptional signature score, which can be evaluated and compared with other prognostic tools in evaluating patients with cancers where Ral has been shown to have a driving role in model systems. In particular, these scores require validation in prospective cohorts and comparison with Ral activation in parallel aliquots of tumor, which, despite the difficulty of biochemical activation assays (8), may become feasible through ELISA or activation state–specific probes. Most importantly, by showing the clinical relevance of Ral in human tumors, our work makes a strong case for investigation of strategies to interrupt Ral function. Irrespective of which therapeutic strategies should succeed, all would benefit from rational cohort selection for clinical trials based on the Ral signature score described herein.

Disclosure of Potential Conflicts of Interest

D. Theodorescu has ownership interest in Key Genomics and is a consultant/advisory board member for KromatID. No potential conflicts of interest were disclosed by the other authors.

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