Contributions of KRAS and RAL in Non–Small-Cell Lung Cancer Growth and Progression

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Introduction: KRAS mutations are poor prognostic markers for patients with non–small-cell lung cancer (NSCLC). RALA and RALB GTPases lie downstream of RAS and are implicated in RAS-mediated tumorigenesis. However, their biological or prognostic role in the context of KRAS mutation in NSCLC is unclear.

Methods: Using expression analysis of human tumors and a panel of cell lines coupled with functional in vivo and in vitro experiments, we evaluated the prognostic and functional importance of RAL in NSCLC and their relationship to KRAS expression and mutation.

Results: Immunohistochemical (N = 189) and transcriptomic (N = 337) analyses of NSCLC patients revealed high RALA and RALB expression was associated with poor survival. In a panel of 14 human NSCLC cell lines, RALA and RALB had higher expression in KRAS mutant cell lines whereas RALA but not RALB activity was higher in KRAS mutant cell lines. Depletion of RAL paralogs identified cell lines that are dependent on RAL expression for proliferation and anchorage independent growth. Overall, growth of NSCLC cell lines that carry a glycine to cystine KRAS mutation were more sensitive to RAL depletion than those with wild-type KRAS. The use of gene expression and outcome data from 337 human tumors in RAL-KRAS interaction analysis revealed that KRAS and RAL paralog expression jointly impact patient prognosis.

Conclusion: RAL GTPase expression carries important additional prognostic information to KRAS status in NSCLC patients. Simultaneously targeting RAL may provide a novel therapeutic approach in NSCLC patients harboring glycine to cystine KRAS mutations.

Key Words: RAL, RAS, Non–small-cell lung cancer, GTPase, Prognosis.

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In the United States, 228,190 new diagnoses and 159,480 lung cancer deaths are projected for 2013.[1] Worldwide lung cancer is also the leading cause of cancer death.[1] Historically, therapy has been guided by tumor histology. Yet, despite recent advances, overall survival in the United States remains 16% at 5 years for non–small-cell lung cancer (NSCLC), a group composed of adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma subtypes. During the past decade, unique genetic changes have been observed in NSCLCs[2–5] and used for both prognostication and therapeutic decision making using targeted agents.

RAS mutations are found in 25% to 40% of NSCLCs,[5–7] with KRAS mutations accounting for 90%. Approximately 97% of KRAS mutations in NSCLC involve codon 12 or 13[8] and are a negative prognostic marker for patients with NSCLC.[9] Unfortunately, direct RAS targeted therapy is not clinically available.[4,5] An alternate strategy is targeting signal proteins downstream of RAS. RAS proteins signal primarily through three cascades; MAPK, PI3K/AKT, and RAL GTPase.[2,5] Inhibitors of the MAPK and PI3K/AKT pathway are in various stages of clinical trials for treatment of NSCLC patients with KRAS mutations.[5] Because no compelling clinical rationale exists for RAL targeting in cancer, no therapies have been developed.

RAL GTPases are critical drivers of human oncogenesis with vital roles in tumor growth and migration in pancreatic, prostate, colorectal, and bladder cancers.[10–13]The RAL GTPase family comprises RALA and RALB paralogs, which share 85% amino acid sequence homology.[14] Despite similar structures and downstream effectors they have differential effects on cancer cell phenotypes in different tumor models.[14] Recently, a KRAS driven NSCLC mouse model showed RAL GTPase is required for tumorigenesis.[15] However, the importance of RAL as a prognostic marker and its functional importance with respect to KRAS expression and mutation status in human NSCLC is unknown.[16] Here we combine for the first time transcriptomic and immunohistochemical (IHC) analyses on human samples with molecular manipulation and evaluation of RAS and RAL in human NSCLC cell lines to show that RALA and RALB are both important prognostic factors in NSCLC and drive tumor growth in vivo. Our work provides the rationale for thinking that simultaneous inhibition of MAPK, PI3K/AKT, and RAL pathways would be effective treatment of patients with KRAS mutations[5] and impetus for drug development directed at the RAL pathway.

MATERIALS AND METHODS

Cell Lines and Biochemical Reagents

NSCLC cell lines H358, H2122, H460, A549, H157, Calu-6, SW1573, H2009, H2228, H1703, HCC4006, Calu-3, H322, and H292 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum as recommended by American Type Culture Collection. Small interfering RNA (siRNA) targeting RALA (siRALA, 5′-GACAGGUUUCUGUAGAAGA-3′), RALB (siRALB, 5′-AAGCUGACAGUUAUAGAAA-3′), or both (siRALA+B, 5′-GACUAUGAACCUACCAAAG-3′) were obtained as previously described.[17] A second set of siRNA against RALA (siRALA II, 5′-CAGAGCUGAGCAGUGGAUU-3′) and RALB (siRALB II, 5′-GGUGAUCAUGGUUGGCAGC-3′) was also used. A nonspecific siRNA (siCTL, 5′-CGTACGCGGAATACTTCGA-3′) was used as control for all the experiments.[17] All siRNAs were from Dharmacon (Lafayette, CO). Cells were transduced with siRNA (200 nmol per liter) by using oligofectamine (Invitrogen) according to the manufacturer's instructions. *KRAS* wild-type (WT) and KRAS G12C and G12V mutant constructs were from the Missouri S&T cDNA Center (Rolla, MO).

Western Blotting, RAL, and KRAS Activation Assays

Cells were lysed using CelLytic™ Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO) and Westerns performed as previously described.[17] Equal amounts of protein were subjected to sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and probed with antibodies against RALA (BD Transduction Laboratories, San Jose, CA), RALB (Millipore, Billerica, MA), α-Tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), extracellular-signal-regulated kinases (ERK) and phospho-ERK (pERK), AKT and phospho-AKT, and KRAS (all from Cell Signaling, Danvers, MA). RALA and RALB activity was measured using RAL Activation Assay Kit (Millipore). In brief, cell lysates were incubated with RALBP1 agarose slurry for 4 hours at 4°C. After this the beads were washed and boiled in Laemmli Sample Buffer. The boiled samples are divided in half and run as two set of samples on SDS-PAGE followed by Western blotting. One set of sample was probed for RALA and the second for RALB.[17] horseradish peroxidase (HRP) labeled mouse or rabbit secondary antibodies (Cell Signaling) were used to develop the blots by chemiluminescence by using enhanced chemoluminescence (ECL) (Pierce, Rockford, IL). KRAS activation assay was carried out on H2228 cells stably transfected with empty vector, KRAS WT, KRAS G12C and G12V constructs by using RAS Activation enzyme-linked immunoabsorbent assay (ELISA) Kit (Millipore) following the manufacturer's protocol.

Cancer Cell Growth In Vitro and In Vivo

For assessment of monolayer proliferation, 10^3 cells were plated in 96 well plates in triplicate or greater 48 hours after the cells were transduced with control siRNA (siCTL) or siRNA targeting RALA, RALB, or both (RALA+B). Cell numbers were determined daily by using the CYQUANT assay (Invitrogen) as directed by the manufacturer. For anchorage independent growth assessment, lines were plated in triplicate in 0.4% agar (15,000 or 20,000 cells/well) 48 hours after siRNA transduction. At selected time points based on the colony-forming capacity of each cell line, colonies formed in soft agar were stained with Nitro-BT (Sigma-Aldrich) at 37°C overnight and counted using software ImageJ.[18] For xenograft experiments, 4-week-old female athymic NCr-*nu*/*nu* mice were obtained from the National Cancer Institute (NCI-Frederick, Frederick, MD). At 6 to 8 weeks of age they were injected with H2122 cells (2×10^5 cells/site) transduced 48 hours earlier with RALA, RALB, RALA+RALB, or control siRNA in their left and right flanks, and monitored for subcutaneous tumor growth. Tumors were measured regularly as indicated in the results and tumor volume calculated as described.[19]

RAL IHC Analysis

Details on primary tumor samples and specific protocols for IHC sample preparation and RAL GTPase staining are in Supplementary Information (Supplementary Digital Content 1, http://links.lww.com/JTO/A482). Membranous and cytoplasmic expression was scored separately, and the association of RALA and RALB expression with patient outcomes (i.e., time to progression, overall survival, etc.) was performed by the University of Colorado Cancer Center Biostatistics and Bioinformatics Shared Resource by using SAS/BASE and SAS/STAT software, Version 9.2 of the SAS System for Windows (SAS Institute Inc., Cary, NC). Cox proportional hazards model was fit and the proportional hazards assumption was checked and found to be met. The interaction between RALA and RALB protein expression was analyzed as well as the association to outcome of each protein alone, adjusting for patient characteristics, stage (1/2/3/4), age, histology (adenocarcinoma, squamous cell carcinoma, NSCLC, not otherwise specified, mixed adenocarcinoma and squamous cell carcinoma characteristics [mixed], and sex (M/F). When analyzing the association between overall survival and the interaction between RALA and RALB, expression scores were dichotomized at their respective medians (i.e., low RALA+low RALB, low RALA+high RALB, high RALA+low RALB, high RALA+high RALB); when analyzing one protein alone, the expression score was categorized by its quartiles (lowest 25%, 25%–50%, 50%–75%, highest 25% of scores)

Microarray Analysis

Three publicly available NSCLC patient data sets (Supplementary Table 3) were used. To examine whether RALA or RALB gene expression could stratify patient survival, patients were divided into two groups according to gene expression or risk scores and were compared by Cox proportional hazards models and log-rank tests (see Supplementary Information, Supplementary Digital Content 1, http://links.lww.com/JTO/A482). To analyze the interaction between KRAS and RAL genes, a patient was classified as either high- or low-expressing by using a gene's median expression. Patient groups with different expression levels of KRAS and

RAL genes were compared by Cox proportional hazards models and log-rank tests.

RESULTS

RAL Expression Stratifies Prognosis in NSCLC Patients

It is well established that in cancerous cells there is more RAL-GTP versus RAL-GDP compared with normal cells and its role in various tumor types is acknowledged.[10,14,17,20] However, RAL expression as a prognostic biomarker in cancers is unclear.[21] The role of RAL expression as a prognostic biomarker in NSCLC is yet to be determined. We addressed this gap in the literature by investigating whether RAL expression determined by either IHC or transcriptomic analysis was prognostic in patients with NSCLC. The clinical cohort used for the prognostic (IHC) study is described in Supplementary Table 1. Analysis of RALA and RALB membrane and cytoplasmic expression in the tumor samples revealed that NSCLC patients have variable degree of RAL GTPase membrane and cytoplasmic expression (Fig. 1*A*, *C*). There is higher cytoplasmic expression of RALA and RALB compared with membrane expression ($p < 0.05$; Fig. 1*B, D*). Also, RALA membrane expression is higher than RALB membrane expression ($p < 0.0001$; Fig. 1*B, D*). Because membrane localization of GTPases is canonically associated with higher activity[14,20,22,23] these data suggest NSCLC specimens harbor more activated RALA than RALB. Interestingly, RAL cytoplasmic and membrane expression does not change as a function of tumor stage and histology (Supplementary Figure 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). With IHC techniques established, the correlation of RALA and RALB membrane and cytoplasmic expression with patient survival was evaluated. High RALA membrane expression (top 25% expression) trended toward poor overall survival when compared with low RALA membrane expression (low 25% expression) though the data are not statistically significant (Fig. 2*A*, Supplementary Table 2) whereas high RALB membrane expression (top 25% expression) was associated with poor overall survival when

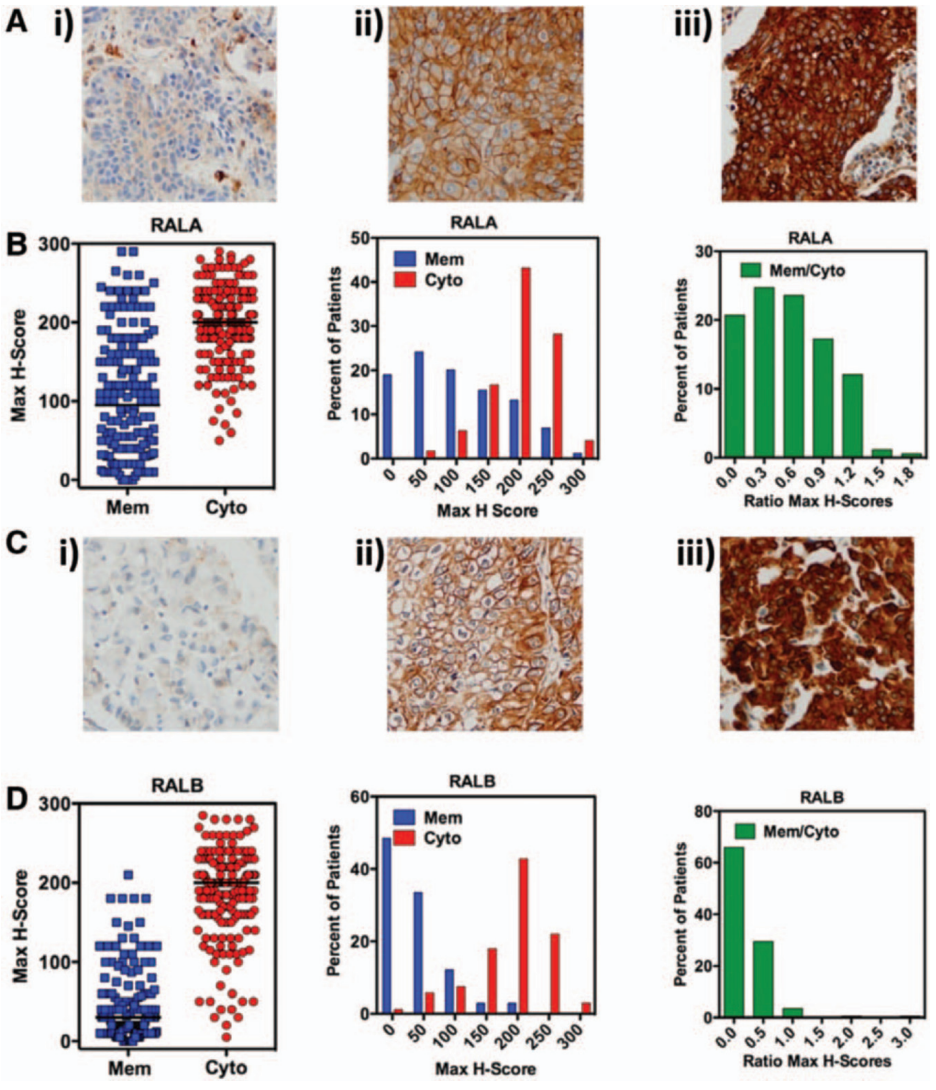

FIGURE 1. RALA and B cytoplasmic and cell membrane immunohistochemical staining in the NSCLC patient tumor samples. H-score was used to measure the staining for RAL protein expression (see Supplementary Materials and Methods section, Supplemental Digital Content 1, http://links.lww.com/JTO/A482). The average H-score of the triplicate cores per patient was highly correlated to the core with the highest score from the same patient, thus further analyses were performed using the maximum H-score. *A* and *C*, Show typical examples of (i) low or no cytoplasmic and membranous protein; (ii) high membranous and low cytoplasmic; (iii) high cytoplasmic and high membranous staining for RALA and RALB. *B* and *D*, Show three graphical depictions of RALA and B membrane and cytoplasmic staining in NSCLC specimens. NSCLC, non–small-cell lung cancer.

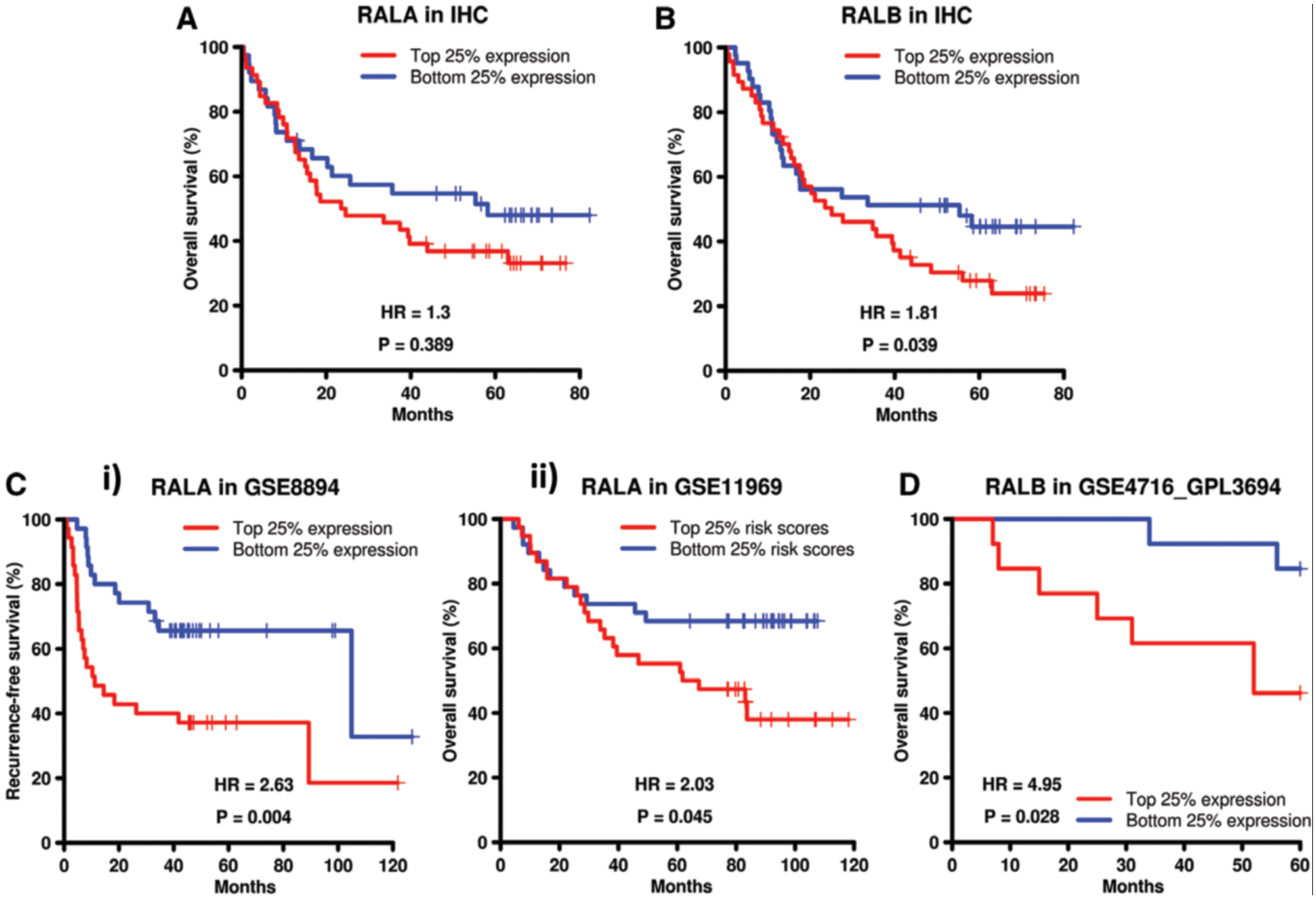

FIGURE 2. RALA and B protein and mRNA expression stratify NSCLC patient survival. Overall NSCLC patient survival as a function of (*A*) RALA and (*B*) RALB membrane expression as evaluated by IHC. *C*, Patient survival in NSCLC data sets GSE8894 and GSE11969 (Supplementary Table 3) as a function of RALA (i) mRNA expression or (ii) risk score (see Materials and Methods section). *D*, Overall patient survival in NSCLC data set GSE4716_GPL3694 as a function of RALB expression. IHC, immunohistochemistry; HR, hazard ratio; NSCLC, non–small-cell lung cancer.

compared with low RALB membrane expression (bottom 25% expression, $p = 0.04$; Fig. 2*B*, Supplementary Table 2). When we evaluated all four quartiles of RALA and B expression and effect on patient survival the data were not found to be statistically significant (Supplementary Figure 2*A, B*, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). Other mathematical constructs of RAL expression (with either each paralog separately or combined) such as membrane (data not shown), membrane/cytoplasm (data not shown), or membrane + cytoplasm (Supplementary Figure 2*C*, *D*, Supplemental Digital Content 2, http://links.lww.com/JTO/A483) among others did not offer enhanced predictive ability over individual analysis.

Next, we examined the prognostic importance of RAL mRNA expression in predicting NSCLC patient survival. Three public gene expression data sets of lung cancer patients were analyzed (Supplementary Table 3). In two of three data sets, patients with high RALA expression had poorer recurrence-free survival and overall survival in comparison to low RALA expression (Fig. 2*C*i and *C*ii). High RALB mRNA expression was also prognostic of poor overall survival in one of three data sets (Fig. 2*D*). When we evaluated all four quartiles of RALA and B expression and impact on patient survival, the data were statistically significant in two of three data sets for RALA (Supplementary Figure 3*A*, *B*, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). RALB expression and impact on patient survival was not statistically significant when we investigate all four quartiles (Supplementary Figure 3*C*, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). However, RAL protein and mRNA expression were a strong prognostic marker of patient survival when we compared patients with very high RAL expression (top 25%) with patients with very low RAL expression (bottom 25%).

Expression and Activation of RAL Is Related to KRAS Mutation in Human NSCLC Cell Lines

We next sought to determine the relationship of RAL expression and activity to the mutation status of its upstream regulator KRAS by evaluating RAL protein expression in

14 human NSCLC cell lines. RALA and RALB expression and activity were detected in all lines (Fig. 3*A*, *C*). Eight cell lines (H358, H2122, A549, H2009, H460, SW1573, H157, and Calu-6) have KRAS mutations whereas the remaining (H292, H322, H1703, H2228, H4006, and Calu3) are KRAS WT, with H4006 harboring an epidermal growth factor receptor mutation. Quantification of expression by densitometry revealed average RALA and RALB expression was higher in KRAS mutant lines compared to WT lines ($p = 0.043$ and 0.036 respectively, Fig. 3*B*). Total RALA activation was also higher in KRAS mutant NSCLC cell lines compared with WT cells ($p = 0.048$, Fig. 3*D*). In contrast, minimal RALB activation was observed in these lines (Fig. 3*C*) with no correlation between total RALB activity and KRAS mutation status (Fig. 3*D*). There was no relationship between RAL paralog expression and their corresponding activation levels ($p = 0.08$ for RALA and $p = 0.23$ for RALB, Supplementary Figure 4, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was also carried out for RALA and B mRNA expression in these cell lines (Supplementary Table 4). Cell lines with KRAS mutations trended toward high RALA (0.013 versus 0.011) and RALB (0.0024 versus 0.0015) relative mRNA expression normalized to β-actin compared with WT lines; however,

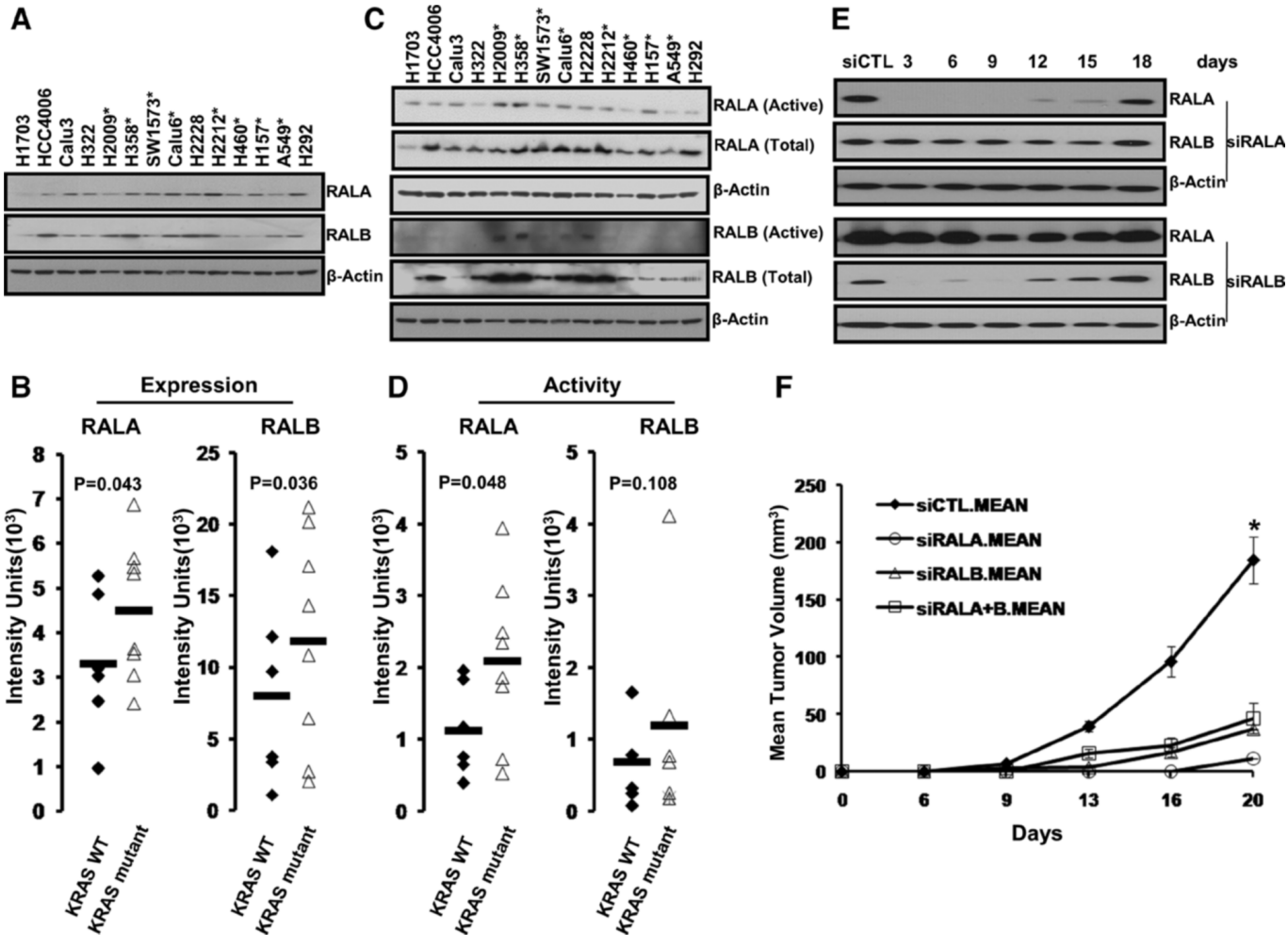

FIGURE 3. RALA and RALB expression and activity in human NSCLC cell lines. *A*, RALA and RALB expression in 14 NSCLC cell lines by Western blot. Cell lines with KRAS mutation indicated with asterisks. *B*, Densitometric quantification of RALA and RALB expression shows their expression is higher in KRAS mutant NSCLC cell lines ($p < 0.05$, Student's *t* test). *C*, RALA and RALB activation levels in the 14 NSCLC cell lines. Cell lines with KRAS mutation indicated with asterisks. *D*, Densitometric analysis revealed higher RALA activation in KRAS mutant NSCLC cell lines ($p < 0.05$, Student's *t* test). No relationship to KRAS was found with RALB. *E*, Knockdown of RAL GTPase in H2122 cells after transient transfection with siRNA against RALA (siRALA) and B (siRALB). Cells were lysed 3, 6, 9, 12, 15, and 18 days after transfection, and RAL GTPase knockdown was determined by Western blot. *F*, Loss of RALA, B or A+B reduced subcutaneous tumor growth in mice. H2122 cells were transfected with siRNA against RALA (siRALA), B (siRALB), and A + B (siRALA+B), injected in mice and studied for in vivo tumor growth (see Materials and Methods section). Luciferase siRNA transfected cells were used as control (siCTL). *$p < 0.05$ by Student's *t* test. NSCLC, non–small-cell lung cancer; WT, wild-type; siRNA, small interfering RNA.

the data were not statistically significant with $p = 0.11$ and 0.28, respectively. We carried out RALA and RALB IHC on the above-mentioned cell lines to study the relationship of RAL activation to RAL membrane and cytoplasmic expression and found no relationship.

RAL Expression Drives NSCLC Growth In Vitro and In Vivo

To determine whether RAL has a functional role in human NSCLC we evaluated the dependency of cell line proliferation and anchorage independent growth on RAL expression. For these experiments we used KRAS WT NSCLC cell lines and cell lines with KRAS mutation in codon 12, the most common KRAS mutation site in NSCLC.[3] RAL GTPase depletion was performed using specific RAL siRNAs targeting RALA, RALB, or a motif common to both RALA and RALB (A+B) leading to simultaneous knockdown.[17] RAL GTPase knockdown was evaluated 72 hours after siRNA transduction by Western blotting for RALA and RALB and identified a 70% to 80% depletion of RALA, RALB, or both proteins in all lines (Supplementary Figure 5, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). Depletion of RALA, RALB, or both resulted in inhibition of anchorage dependent and independent growth of four of six KRAS WT and five of six KRAS mutant NSCLC cell line ($p < 0.05$, Table 1). Loss of RALA versus RALB versus both proteins had different degrees of growth inhibition. Depletion of RALA had the greatest effect, with inhibition of monolayer growth in two of six KRAS WT NSCLC cell lines compared with five of six KRAS mutant cell lines ($p = 0.037$) and inhibition of anchorage independent growth in one of six KRAS WT cell lines compared with four of six KRAS mutant cell lines ($p = 0.04$).

The impact of RAL depletion on anchorage independent growth was greatest on the H358 (G12C KRAS) and H2122 (G12C KRAS) cell lines (Table 1). H2122 had near-complete inhibition of colony formation with RALA knockdown whereas knockdown of RALB or both proteins resulted in 90% and 79% inhibition. H358 had 38%, 27%, and 83% decrease in anchorage independent growth with similar RAL depletions (Table 1). To confirm these observations were not because of nonspecific siRNA effects, the study was repeated in H2122 with a second set of RAL GTPase siRNAs (siRALA II and siRALB II) and similar results were observed (Supplementary Figure 6, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). Interestingly, investigation of the PI3K/AKT and MAPK pathways, which also signal downstream of KRAS, revealed that these alternate pathways had minimal to no activation in NSCLC cell lines that carry the KRAS G12C mutation (Supplementary Figure 7, Supplemental Digital Content 2, http://links.lww.com/JTO/A483).

The H2122 cell line was then used to study RAL's effect on tumor growth in vivo. H2122 cells were transfected with control, RALA, RALB, or RALA+RALB siRNAs and RAL expression evaluated at multiple time points after transfection (Fig. 3*E*). RAL expression was found to be suppressed for up to 15 days (Fig. 3*E*) supporting the notion that in vivo tumor growth would be suppressed during this time frame. With these data in hand, we repeated the depletion in H2122 cells and inoculated them into mice 2 days after transfection. As shown in Figure 3*F*, transient knockdown of RALA, RALB, or both proteins had significant impact on tumor growth

TABLE 1. Monolayer and Anchorage-Independent Growth of NSCLC Cell Lines with Knockdown of RAL GTPase

			RALA Knockdown		RALB Knockdown		RALA+B Knockdown	
Cell Line	**Histology**	***KRAS***	**Monolayer Growth**	**Anchorage-Independent Growth**	**Monolayer Growth**	**Anchorage-Independent Growth**	**Monolayer Growth**	**Anchorage-Independent Growth**
H322	Adenocarcinoma	WT	−52%*	−41.2%*	−33%*	−42.3%*	−46%*	−39%*
H1703	Adenocarcinoma	WT	N.S.	N.S.	N.S.	N.S.	N.S.	−52.3%*
H2228	Adenocarcinoma	WT	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
H292	Mucoepidermoid	WT	N.S.	No colony	−23%*	No colony	N.S.	No colony
H4006	Adenocarcinoma	WT	N.S.	No colony	N.S.	No colony	N.S.	No colony
Calu3	Adenocarcinoma	WT	−33%*	No colony	−36%*	No colony	−39%*	No colony
H2122	Adenocarcinoma	G12C	−91%*	−97%*	−61%*	−89.2%*	−42%*	−79.6%*
H358	Adenocarcinoma	G12C	−24%*	−38%*	N.S.	−27.6%*	−86%*	−83.4%*
A549	Adenocarcinoma	G12S	−26%*	−24.5%*	−29%*	N.S.	−27%*	−51.3%*
H2009	Adenocarcinoma	G12A	−25%*	−35%*	−19%*	−28.7%*	28%*	−15.8%*
H157	Squamous cell	G12R	−24%*	N.S.	−19%*	N.S.	N.S.	N.S.
SW1573	Alveolar cell	G12A	N.S.	No colony	N.S.	No colony	N.S.	No colony

Cells were transfected with siRNA against RAL GTPase and plated for proliferation and anchorage-independent growth assay (see Materials and Methods section). Monolayer growth was measured 4 days after transfection with results representing the mean of 2–3 studies with 4–5 replicates per cell line. Anchorage-independent growth results are mean of three experiments. (+) indicates increase compared with control. (−) indicates decrease compared with control. "N.S." is no significant change. "No colony" indicates cells did not exhibit anchorage-independent growth.

NSCLC, non–small-cell lung cancer; WT, wild-type; siRNA, small interfering RNA.

*$p < 0.05$ (Student's t test).

with a 94%, 81%, and 81% decrease, respectively, at 20 days ($p < 0.05$). Together, these data suggest that RAL expression is especially important for tumor growth in NSCLC cell lines harboring G12C KRAS mutations.

G12C KRAS Mutation Regulates Anchorage Independent Growth through RAL

Functional assays revealed cell lines with KRAS G12C mutation were most dependent on RAL GTPase for tumor processes (proliferation, anchorage independent growth; Table 1). Therefore, to determine whether KRAS G12C mutation results in tumor progression that is specifically dependent on the RAL pathway, H2228 cells were stably transfected with KRAS WT, KRAS G12C mutant (found in H2122), and KRAS G12V mutant (Fig. 4*A*). The H2228 cell line was chosen because it is KRAS WT, has high RALA and B expression but its anchorage independent growth is independent of RAL expression (Table 1). H2228 cells overexpressing KRAS G12C and G12V mutants have high KRAS activation compared with cells transfected with KRAS WT (Fig. 4*B*). An increase in RAL activation in H2228 cells overexpressing the KRAS G12C mutant was also seen when compared with H2228 cells overexpressing KRAS WT and KRAS G12V mutant (Fig. 4*C, D*), suggesting increased signaling through RAL in cells having KRAS G12C mutation. Importantly, KRAS G12C and G12V overexpressing cells had a 48% and 127% increase in anchorage independent growth compared with KRAS WT ($p < 0.05$), respectively. This increase in anchorage independent

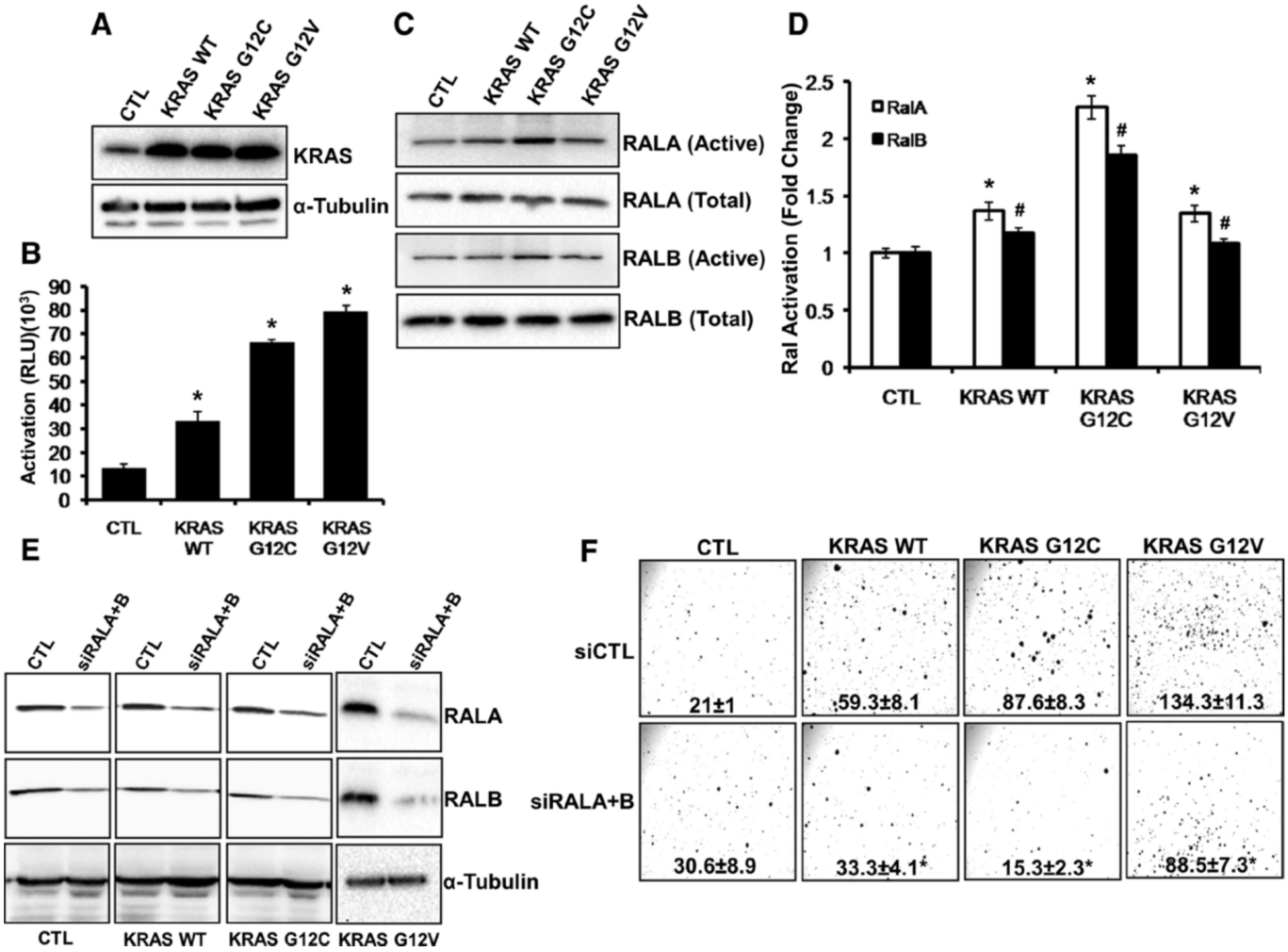

FIGURE 4. The role of RAL and KRAS in tumor growth. *A*, KRAS expression in H2228 cells stably transfected with empty vector (CTL), KRAS WT, KRAS G12C and G12V mutants (KRAS G12C and KRAS G12V) detected by Western blot. *B*, KRAS activation in the engineered H2228 cell lines as determined by *RAS* activation enzyme-linked immunosorbent assay (see Materials and Methods section). *$p < 0.05$ by Student's *t* test. *C*, RALA and B activation observed by Western blot in H2228 cells transfected with empty vector (CTL), *KRAS* WT, and KRAS G12C and G12V mutants (KRAS G12C and KRAS G12V) (see Materials and Methods section). *D*, Densitometric analysis of RALA and B activation observed in (*C*). Asterisk and pound indicate $p < 0.05$ for RALA and B by Student's *t* test. *E*, RALA and B loss observed by Western blot after transfection of engineered H2228 cell lines with siRNA targeting both RALs (siRALA+B). A luciferase siRNA transfected cells are used as control (siCTL). *F*, Anchorage independent growth of engineered H2228 cell lines after loss of RAL GTPase (*$p < 0.05$ by Student's *t* test). WT, wild-type; siRNA, small interfering RNA.

growth for cells overexpressing KRAS G12C was RAL dependent, as shown by a 83% inhibition in anchorage independent growth with siRNA-mediated depletion of RALA+RALB in H2228 cells expressing KRAS G12C compared with 44% and 34% inhibition in cells overexpressing WT KRAS and KRAS G12V mutant (Fig. 4*E, F*). This suggests that NSCLC tumors with a KRAS G12C mutation become more reliant on the RAL pathway for tumor growth.

Contributions of KRAS and RAL in NSCLC Tumor Progression

These data suggest RAL expression mediates KRAS-driven NSCLC growth. This extends prior work showing RAL is required for KRAS-induced tumor formation.[20] To determine whether this relationship is supported by clinical data, we examined the relationship of RAL paralog expression to that of KRAS mRNA expression in regard to survival of NSCLC patients. In data set GSE8894 where we found high RALA mRNA predicted poor patient outcome (Fig. 2*C*), stratification of patients by KRAS and RALA mRNA expression status found high KRAS and RALA mRNA expression was associated with poor recurrence-free survival compared with patients with high KRAS and low RALA mRNA expression (p = 0.031; Fig. 5*A*). In addition, analysis of data set GSE11969 where we had previously found high RALA risk score predicted poor patient outcome (Fig. 2*C*) suggested that KRAS risk score impacts the prognostic stratification driven by the RALA risk score with high RALA and KRAS mRNA expression now associated with poor overall survival compared with patients with high RALA and low KRAS mRNA expression (p = 0.022; Fig. 5*B*). These analyses indicate that KRAS and RALA can contribute independently to patient prognosis despite their canonical hierarchical relationship. A similar analysis was carried out for KRAS and RALA in GSE4716_GPL3694 in which high RALB mRNA expression predicted poor overall patient survival (Fig. 2*D*) but none of the interactions were statistically significant.

We also evaluated the interaction of RALB and KRAS expression in the two data sets where high RALA mRNA (GSE8894) or risk score (GSE11969) is predictive of poor patient outcome but no interaction was observed. However, in data set GSE4716_GPL3694 patients with high KRAS and RALB mRNA expression had poor overall survival compared with patients with high KRAS and low RALB mRNA expression (p = 0.028; Fig. 5*C*). Patients with high KRAS and RALB mRNA expression trended (p = 0.082) toward poor overall survival compared with patients with low KRAS and high RALB mRNA expression (Fig. 5*D*). Because KRAS mutation status of patients in these data sets was unknown, we could not investigate the survival information carried by RAL GTPase in patients carrying KRAS mutations.

DISCUSSION

Recently RAL GTPase null and conditional knockout mice were crossed with mice developing KRAS-driven NSCLC to show that RAL is important in KRAS-driven pulmonary

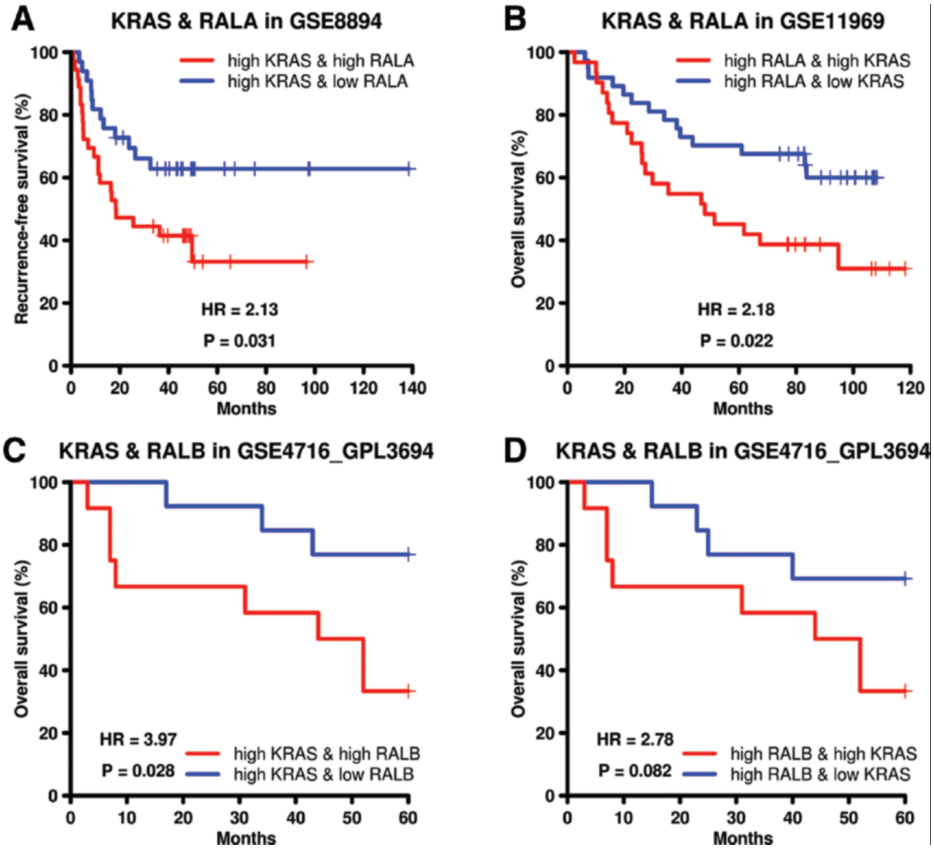

FIGURE 5. RAL and KRAS mRNA expression and risk score stratify overall and recurrence-free survival in NSCLC patients. *A*, Recurrence-free survival in NSCLC data set GSE8894 as a function of RALA mRNA expression in patients with high KRAS mRNA expression. *B*, Patient overall survival in NSCLC data set GSE11969 as a function of KRAS risk score in patients with high RALA risk score. *C*, Patient overall survival in NSCLC data set GSE4716_GPL3694 as a function of RALB mRNA expression in patients with high KRAS mRNA expression. *D*, Patient overall survival in NSCLC data set GSE4716_GPL3694 as a function of KRAS mRNA expression in patients with high RALB mRNA expression. NSCLC, non–small-cell lung cancer; HR, hazard ratio.

tumorigenesis.[15] PI3K/AKT and MAPK pathways are also important for KRAS-driven tumors.[5] Here we move the field forward by using IHC, transcriptomic and human cell line–based functional analysis to investigate the prognostic information carried by RAL GTPase and its role in NSCLC tumor progression as a function of KRAS. We began our investigations by evaluating the prognostic information carried by RAL protein and mRNA expression in human cancers. IHC analysis of RAL protein expression revealed that RALB cell membrane expression is a negative prognostic marker in NSCLC (Fig. 2*B*). Because membrane localization suggests the GTPase is in an active state[14,20,22,23] we suggest that active RALB is a prognostic marker for decreased survival in NSCLC. In addition, patients with high RALA membrane expression trended toward poorer overall survival but the data were not statistically significant (Fig. 2*A*). Because RAL cytoplasmic expression (data not shown) and total RAL expression (Supplementary Figure 2*C*, *D*, Supplemental Digital Content 2, http://links.lww.com/JTO/A483) are not predictive of survival we can advocate that membranous RAL is the functionally active form of RAL in NSCLC patient tumors. This protein analysis was followed up with an evaluation of RALA and RALB mRNA expression where higher levels were associated with shorter recurrence-free and overall survival (Fig. 2*C, D*). Interestingly, we noted that in the patient data sets (IHC or microarray) either RALA or RALB primarily carried the prognostic information (Fig. 2). We conclude that RAL carries important clinical information. However, we speculate that in each tumor only one of the two RAL paralogs is functionally dominant.

Investigation of the functional role of RAL GTPase in NSCLC revealed that loss of RALA, RALB, or both resulted in variable changes in monolayer proliferation, anchorage independent growth, and subcutaneous tumor formation in NSCLC cell lines (Table 1, Fig. 3*E, F*). RALA expression seemed more important in driving growth in KRAS mutant NSCLC cell lines compared with KRAS WT cell lines, suggesting that in this panel of NSCLC cell lines RALA is the functionally dominant isoform driving KRAS-dependent tumor growth (Table 1, Fig. 3*A, B*). Interestingly, cell lines with a G12C KRAS activating mutation had greater dependence on RAL for anchorage independent growth compared with lines with other codon 12 mutations or KRAS WT (Table 1, Fig. 4). Furthermore, NSCLC cell lines (H2122 and H358) with the G12C mutation had minimal activation of PI3K/AKT and MAPK pathways downstream of RAS (Supplementary Figure 7, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). H2122 has no activation whereas H358 has minimal activation of these alternate pathways and high activation of RAL GTPase. The lack of alternate pathway activation in these cell lines suggested that they are dependent on RAL signaling for their tumorigenic phenotype whereas those cell lines with activation of PI3K/AKT and MAPK pathways downstream of KRAS are partially dependent or not dependent on RAL GTPase because of the availability of these alternate pathways for tumor progression (Table 1). A549 was the only exception to this trend (Supplementary Figure 7, Supplemental Digital Content 2, http://links.lww.com/JTO/A483). This line does not have activation of PI3K/AKT and MAPK pathways yet is only marginally dependent on RAL expression for tumorigenicity (Table 1). This may be explained by the finding that A549 is independent of RAS activity for tumor growth.[24] We gained further support for the notion that KRAS G12C mutations drive tumor progression via RAL GTPase by stably overexpressing KRAS G12C mutant construct in H2228 NSCLC cell line, which is KRAS WT, and showing RAL loss inhibits anchorage independent growth (Fig. 4). Our findings confirmed and validated initial observations made by Ihle et al.[16]

To understand the functional correlation between RAL paralogs and KRAS in NSCLC we examined RAL–KRAS statistical interactions in microarray data sets. This analysis was limited by the lack of KRAS mutation status and thus we made the assumption that tumors with high KRAS expression had concomitantly elevated activation. Our analysis revealed that both KRAS and RAL expression are determinants of patient prognosis and suggest that RAL function in NSCLC is driven only in part by KRAS whereas KRAS also activated other prognostic pathways (Fig. 5). This observation is consistent with the aforementioned finding in the NSCLC cell line panel where we observed activation of PI3K/AKT and ERK pathways in cells that were less dependent on RAL expression (Table 1, Supplementary Figure 7, Supplemental Digital Content 2, http://links.lww.com/JTO/A483).

These studies have significant implications for human NSCLC, such as the identification of their RAL dependence, the identification of RAL as a potential therapeutic target in this disease, and the ability to stratify patients for future anti-RAS or anti-RAL therapy by virtue of RAS and RAL expression and mutation status. Because inhibitors of downstream RAS signaling pathways such as PI3K/AKT and MAPK pathways are in clinical investigation in KRAS mutant NSCLC,[5] these studies serve as a strong impetus for the development of anti-RAL therapeutics to suppress KRAS-driven signal propagation and improve poor clinical outcomes seen in NSCLC patients.

AUTHORS' CONTRIBUTIONS

Overall conception of the project was taken care of by D. Theodorescu. The experiments were designed by S. Guin, F. Hirsch, J.A. Kern, and D. Theodorescu. The entire cell based and xenograft experiments were conducted by S. Guin with help from R. Mishra and C. Owens. qRT-PCR on the cell lines was carried out by V. Vasu. Patient data set analysis was conducted by Y. Ru. Immunohistochemical staining and scoring was overseen by M. Wynes. Immunohistochemical data interpretation and statistical analysis were done by X. Lu and A.E. Barón.

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