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RESEARCH ARTICLE

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RASA3 is a candidate gene in sickle cell disease-associated pulmonary hypertension and pulmonary arterial hypertension

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Abstract

Pulmonary hypertension (PH) is associated with significant morbidity and mortality. RASA3 is a GTPase activating protein integral to angiogenesis and endothelial barrier function. In this study, we explore the association of RASA3 genetic variation with PH risk in patients with sickle cell disease (SCD)-associated PH and pulmonary arterial hypertension (PAH). *Cis*-expression quantitative trait loci (eQTL) were queried for *RASA3* using whole genome genotype arrays and gene expression profiles derived from peripheral blood mononuclear cells (PBMC) of three SCD cohorts. Genome-wide single nucleotide polymorphisms (SNPs) near or in the *RASA3* gene that may associate with lung RASA3 expression were identified, reduced to 9 tagging SNPs for RASA3 and associated with markers of PH. Associations between the top RASA3 SNP and PAH severity were corroborated using data from the PAH Biobank and analyzed based on European or African ancestry (EA, AA). We found that PBMC RASA3 expression was lower in patients with SCD-associated PH as defined by echocardiography and right heart catheterization and was associated with higher mortality. One eQTL for RASA3 (rs9525228) was identified, with the risk allele

correlating with PH risk, higher tricuspid regurgitant jet velocity and higher pulmonary vascular resistance in patients with SCD-associated PH. rs9525228 associated with markers of precapillary PH and decreased survival in individuals of EA but not AA. In conclusion, *RASA3* is a novel candidate gene in SCD-associated PH and PAH, with RASA3 expression appearing to be protective. Further studies are ongoing to delineate the role of RASA3 in PH.

KEYWORDS
pulmonary arterial hypertension, RASA3, sickle cell disease-associated pulmonary hypertension

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive, fatal disease, affecting 15–50 persons per million in the United States and Europe, with poorly understood pathogenic mechanisms and no curative treatments available.[1,2] Given that time from symptom onset to diagnosis may take from 17 to 34 months,[3,4] improved disease awareness and testing are needed. Noninvasive biomarkers are a promising diagnostic option, given their easy access and potential for risk stratification, targeted disease treatment, and monitoring response to therapy.[4,5]

Sickle cell disease (SCD), one of the most common inherited monogenetic diseases, affects millions of individuals worldwide and around 100,000 individuals in the United States.[6] Patients with SCD have chronic hemolysis, a known risk factor for the development of pulmonary hypertension (PH),[7–9] and may develop diastolic dysfunction, renal disease, sleep-disordered breathing, or recurrent thromboembolic disease, all of which can contribute to the development of SCD-associated PH.[5,10] Retrospective studies have found the prevalence of SCD-associated PH to be between 6% and 11% using the former definition of PH of a mean pulmonary artery pressure (mPAP) ≥25 mmHg.[11,12] Using the current definition of PH of an mPAP >20 mmHg, the prevalence of SCD-associated PH is likely considerably higher.

Treatment options are poor for patients with SCD-associated PH. Depending on the profile and severity of their hemodynamics, patients with SCD are often treated similarly to patients with PAH. However, such treatment is extrapolated from guidelines for patients with PAH or based on expert consensus.[13] The discovery of new candidate genes and pathways that could provide novel insights into disease pathogenesis or serve as risk stratification and prognostic tools is desirable. SCD-associated PH shares multiple pathogenic pathways with PAH [5,9,10,13–16] and it is likely that common disease susceptibility candidate genes are shared between these conditions.

RASA3 is a ubiquitously expressed GTPase-activating protein that inhibits R-Ras and Rap1 activity.[17–21] Global loss of RASA3 expression in mice is embryonically lethal due to failure of vasculogenesis and severe bleeding.[22] Conditional endothelial cell-specific knockout of RASA3 in mice recapitulates failure of vascular lumen formation and decreased vascular complexity. In vitro, loss of RASA3 leads to decreased endothelial cell adhesion turnover, decreased migration and permeability, thought to be mediated by hyperactivation of Rap1.[20] Given its multiple roles, as well as its downstream targets which have been implicated in the pathogenesis of PAH, RASA3 may be a critical regulator in the development of PAH. However, no prior work has been done evaluating the role of RASA3 within the context of pulmonary vascular disease. Here we explore the association of RASA3 genetic variation with PH risk in three cohorts of patients with SCD-associated PH and sought to investigate our findings within a PAH cohort.

METHODS

Study subjects

The study was approved by the institutional review boards of the participating institutions and all subjects provided written informed consent. The University of Illinois at Chicago (UIC) cohort contained 178 subjects with hemoglobin SS, 40 with hemoglobin SC, 18 with hemoglobin Sβ^+ thalassemia, 5 with hemoglobin Sβ° thalassemia, and 1 with hemoglobin SO. Ninety-four individuals with elevated tricuspid regurgitation velocity (TRV) and/or clinical

suspicion of PH underwent right heart catheterization (RHC). Ten individuals had precapillary PH defined as an mPAP >20 mmHg and pulmonary capillary wedge pressure (PCWP) ≤ 15 mmHg; nine had post-capillary PH, defined as an mPAP >20 mmHg with a PCWP > 15 mmHg. Seventy-five individuals did not have PH based on an mPAP <20 mmHg. Absence of PH was defined as mPAP <20 mmHg if RHC was performed or TRV < 2.5 m/s if catheterization was not performed.

The Pulmonary Hypertension and the Hypoxic Response in SCD (PUSH) Cohort was recruited from four tertiary medical centers in the United States, with 396 individuals 3–20 years of age screened with echocardiography as outpatients at routine visits. Given their young age, none of these individuals underwent RHC for further evaluation of their pulmonary hemodynamics.

The Walk-Treatment of Pulmonary Hypertension and Sickle cell disease with Sildenafil Therapy (Walk-PHaSST) Cohort was comprised of individuals ≥12 years old with SCD screened with echocardiography as outpatients at routine visits at nine US Centers and one UK Center, with 89.3% of patients over 20 years of age. Patients recruited at UIC were excluded from this analysis to avoid overlap with the UIC cohort. A subgroup of 193 patients with elevated TRV underwent RHC, with pre- and postcapillary PH defined as above. Eight individuals had precapillary PH and three had postcapillary hypertension.

The National Biological Sample and Data Repository for Pulmonary Arterial Hypertension (PAH Biobank) is an NIH-funded consortium of 37 different centers across the United States collecting data from over 3000 individuals with PAH (www.pahbiobank.org). Data were collected from individuals defined as idiopathic or heritable PAH between October 2012 and February 2018 from 36 enrolling centers. Only individuals over 18 years of age were included in this cohort. Patients were recruited prospectively, approved by each of the individual institution's institutional review board, and patients were provided with written informed consent on enrollment. Enrollment criteria included consecutively screened patients who met expanded hemodynamic criteria of mPAP ≥25 mmHg at rest (30 mmHg with exercise), PCWP ≤ 18 mmHg (as defined by REVEAL registry[23]), and pulmonary vascular resistance (PVR) ≥ 3 Wood units (WU). Baseline demographic, clinical, and hemodynamic data were extracted from the available clinical data at the time of RHC as previously described.[24] Ancestry data were self-identified at the time of enrollment (Supporting Information: Figure 1). Medication history use at the time of consent, including the use of PAH medications such as prostacyclin infusion analogs was also captured.[25]

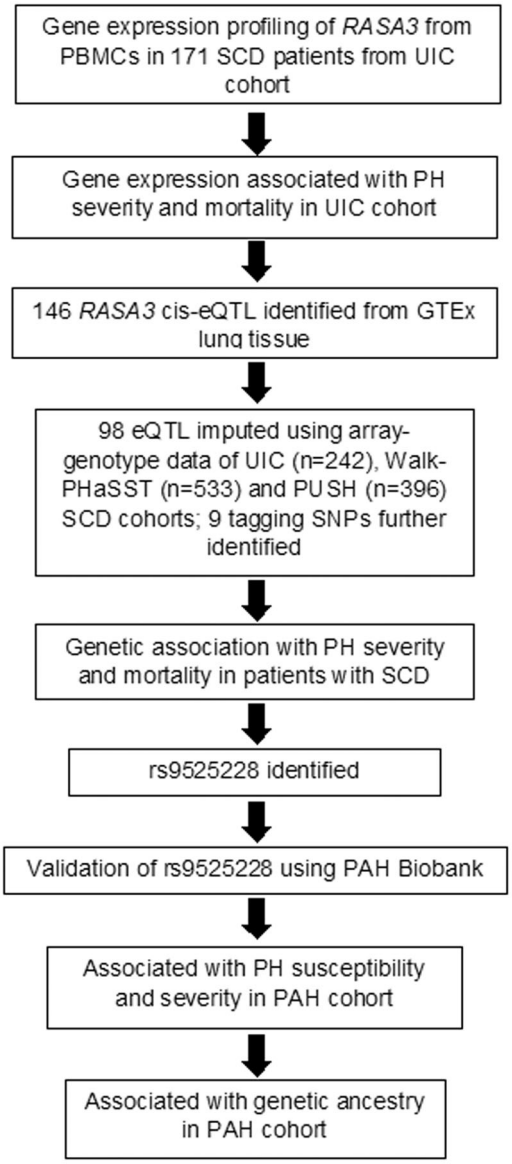

FIGURE 1 Study overview. eQTL, expression quantitative trait loci; PAH, pulmonary arterial hypertension; PBMC, peripheral blood mononuclear cell; PH, Pulmonary hypertension; SCD, sickle cell disease; SNP, single nucleotide polymorphism.

Study strategy

The study was designed similarly to previous studies published by members of our group to compare clinical manifestations and peripheral blood mononuclear cell (PBMC) genomic profiles of subjects with SCD.[16,26,27] We analyzed the correlation between PBMC expression of RASA3 and SCD-associated PH within the UIC cohort.

We then assessed the genetic association between expression quantitative trait loci (eQTL) for *RASA3* identified in lung tissues from the Genotype-Tissue Expression (GTEx) project, and PH phenotypes in SCD patients from the UIC, Walk-PHaSST, and PUSH cohorts. We then sought to investigate whether our findings were also applicable to a cohort of individuals with PAH using data from the PAH Biobank and to associate results with individuals' ancestry. The study scheme is included in Figure 1.

Array gene expression profiling and data processing

Messenger RNA isolated from PBMCs was profiled using Affymetrix Human gene 2.0 ST array as described previously.[28] Probe sequences were aligned to human genotype assembly GRCh37 allowing ≤1 mismatches[29] to select those with perfect unique match. Probes that interrogated multiple genes or that contained single nucleotide polymorphisms (SNPs) with ≥1% minor allele frequency (MAF) in 1000 genomes data of European or African populations were removed. Annotation was based on Gencode release 19. Probe level intensities were $\log^2$ transformed, background corrected,[30] and quantile normalized.[31] Probe intensity was subtracted by the corresponding probe mean across samples. Gene-level expression intensities were summarized as mean probe intensity within each gene.

Array genotyping and data processing

Genomic DNA isolated from PBMCs was labeled and hybridized to the Illumina Human 610-Quad SNP array for Walk-PHaSST and PUSH cohorts and the Affymetrix Axiom genome-wide Pan-African array for the UIC cohort. Genotyping and data preprocessing was as previously described.[16] SNP genotypes were phased[32] and imputed[33] to 1000 genomes project phase 3 data using African reference panels.

To assess ancestry of the SCD cohorts, array genotypes of 199,391 autosomal SNPs available in all three cohorts were combined with 1000 genomes phase 3 data. The SNPs were pruned to 135,921 SNPs by pairwise $r^2 > 0.3$. Principal components analysis was subsequently applied using Plink version 1.9.[34]

Among 146 lung eQTL for *RASA3* obtained from single-tissue eQTL of GTEx release 8,[35] 98 SNPs with dosage $r^2 > 0.9$ and expected effective minor allele count >5 within each of the three SCD cohorts were extracted. The 98 SNPs were pruned to 9 tagging SNPs to reduce the burden of multiple comparison. SNP pruning used a greedy algorithm[36]: (1) within the locus under investigation, the SNP having the most correlated SNPs (linkage disequilibrium $r^2 > 0.3$ was identified as a tagging SNP; (2) the tagging SNP and its correlated SNPs were removed; (3) steps 1 and 2 were repeated for the remaining SNPs until all tagging SNPs were identified.

Genetic association of RASA3 eQTL with clinical phenotypes

Genetic association of the RASA3 eQTL with clinical phenotypes was performed using linear regression for continuous phenotypes and logistic regression for binary phenotypes in the UIC, PUSH, and Walk-PHaSST SCD cohorts with an additive genetic model. Age, gender, severity of sickle genotype (SS, Sβ° and SO Arab vs. SC and Sβ$^+$), hydroxyurea treatment, and population stratification were included as covariates. Bonferroni-corrected $p < 0.05$ (nominal $p < 0.0056$) was considered statistically significant.

Using logistic regression of whole genome genotyping arrays from PAH Biobank samples, we tested single marker variants for genetic association with a diagnosis of PAH and adjusted for sex, age, and 10 principal components as previously described.[27] PAH outcomes were associated with indices of severity as well as self-reported race/ethnicity.[25]

Statistical analysis of clinical data and gene expression variation

Linear regression of clinical outcomes was used for gene expression levels of RASA3 adjusting for age, gender, severity of sickle genotype, and hydroxyurea treatment. Proportional hazards (Cox) regression was used to study relationships between covariates of interest and mortality. The time-to-event outcome analyzed was vital status from blood draw until death or completion of the study, and determined by a combination of social security death index, follow-up calls, and review of electronic medical records. The risk ratio (hazard ratio [HR]) and 95% confidence interval (CI) for each predictor were determined and Kaplan–Meier survival curves were calculated. All analyses were performed using R (version 3.2.0) software (https://www.r-project.org/) and Prism.

TABLE 1 Baseline characteristics of sickle cell disease cohorts.

	PUSH ($n = 396$)	UIC ($n = 242$)	Walk-PHaSST ($n = 533$)
Age (SD)	13.0 (7.0–17.0)	35.0 (27.0–46.0)	35.3 (25.0–46.7)
Female (%)	164 (48%)	144 (60%)	283 (54%)
HbSS/HbSβ^0 (%)	264 (78%)	183 (76%)	403 (77%)
Hydroxyurea (%)	131 (39%)	120 (50%)	182 (35%)
Recent transfusion (%)	63 (20%)	[a]	164 (32%)
TRV (SD)	2.30 (2.11–2.45)	2.41 (2.20–2.66)	2.53 (2.30–2.79)
TRV ≥ 2.5 m/s (%)	30 (9.7%)	67 (46%)	280 (60%)
RHC	[a]	94 (39%)	193 (36%)
PH (%)	[a]	19 (7.8%)	11 (2.1%)
Precapillary PH (%)	[a]	10 (4.1%)	8 (1.5%)
Postcapillary PH (%)	[a]	9 (3.7%)	3 (0.56%)

Abbreviations: HbSβ0, sickle cell-β thalassemia; HbSS, sickle cell disease; PH, pulmonary hypertension; RHC, right heart catheterization; TRV, tricuspid regurgitation jet velocity.

[a]Unavailable data.

RESULTS

RASA3 expression decreased in subjects with SCD-PH and associated with disease severity and prognosis

Baseline characteristics of the three different SCD cohorts are presented in Table 1. The PUSH cohort was composed entirely of children (under the age of 20), and the UIC and Walk-PHaSST cohorts were comprised primarily of young adults. Over three-quarters of individuals had hemoglobin SS or hemoglobin β-thalassemia, and substantial morbidity was suggested by the need for transfusions. Given the young age of the PUSH cohort, none of these individuals underwent RHC for further diagnostic evaluation. A TRV ≥ 2.5 m/s correlates to a right ventricular systolic pressure of approximately 30 mmHg; in adult patients with SCD, a TRV ≥ 2.5 m/s has been associated with chronic hemolysis, resistance to hydroxyurea therapy and increased risk of death.[9] Among children under the age of 20, a TRV ≥ 2.6 m/s was correlated with markers of hemolysis and hypoxia, both of which may contribute to the development of PH.[37] Patients from the three cohorts were all African Americans with variable percentages of European ancestry (Suporting Information: Figure 2).

Using PBMC data from the UIC cohort, estimated systolic pulmonary artery pressure categories, PH (mPAP >20 mmHg, PVR > 3 WU) and PVR were correlated with RASA3 expression and are presented in Figure 2. RASA3 expression was significantly lower in subjects with a TRV ≥ 2.5 m/s. Using RHC data, RASA3 expression was significantly lower in individuals with RHC-confirmed PH compared with individuals without PH. Prior studies have suggested that a PVR ≥ 2 WU is abnormal in individuals with SCD, given a high baseline cardiac output and low vascular resistance.[13,38] However, the most recent European Society of Cardiology and European Respiratory Society guidelines indicate that a PVR > 2 is abnormal.[39] Considering this updated definition and underlying high-output cardiac state of patients with SCD, we used various cutoffs of PVR, and found that RASA3 expression was lower in individuals with higher PVR using a cutoff of ≥1.5, ≥2, or ≥3 WU. Individuals who died during follow-up had significantly lower RASA3 expression, a trend that persisted over 12 years of follow-up (Cox model HR 0.13, 95% CI 0.028–0.6, $p = 0.0087$, Figure 3).

Association of RASA3 eQTL rs9525228 with PH susceptibility in SCD

We did not find significant eQTL for *RASA3* using the PBMC expression data of the UIC cohort (data not shown). We, therefore, examined 146 *RASA3* eQTL identified in lung tissues from the GTEx project. We extracted 98 eQTL and reduced the number of association tests by selecting nine SNPs using a greedy

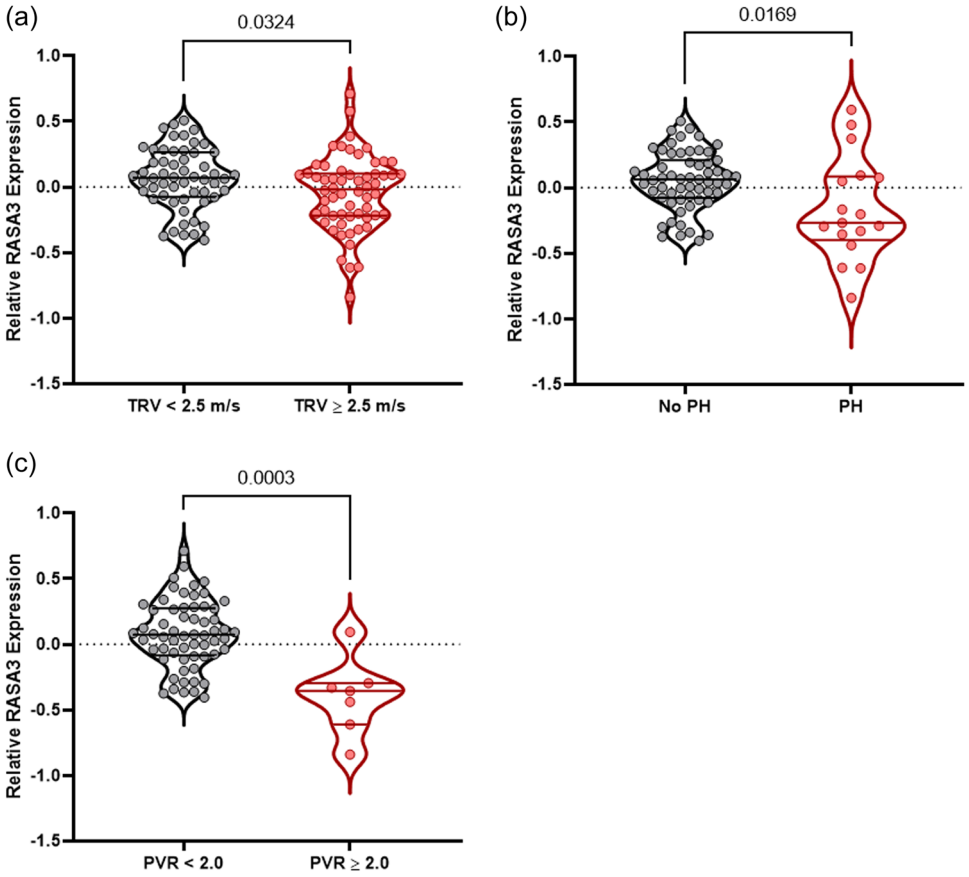

FIGURE 2 RASA3 expression is lower using different measures of PH severity in SCD. (a): TRV ≤ 2.5 m/s $n = 56$, TRV > 2.5 m/s $n = 60$. (b): No PH $n = 59$, PH $n = 17$. (c): PVR < 2 $n = 61$, PVR ≥ 2 $n = 7$. PH, pulmonary hypertension; PVR, pulmonary vascular resistance (Wood units); TRV, tricuspid regurgitation jet velocity.

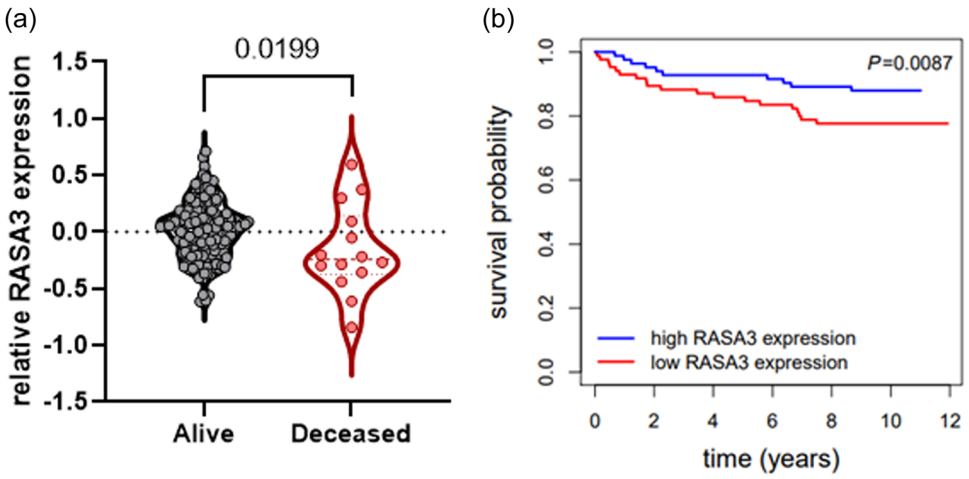

FIGURE 3 Lower RASA3 Expression is associated with higher mortality among patients with sickle cell disease. (a) Eighty-five individuals were characterized as having high RASA3 expression, and 83 individuals were characterized as having low RASA3 expression. (b) At the end of follow-up, 75 individuals with high RASA3 expression and 64 individuals with low RASA3 expression were still living.

algorithm. These nine SNPs for *RASA3* eQTL were associated with TRV ≥ 2.5 m/s or RHC-confirmed PH. Ultimately we identified that the T allele of rs9525228, an intronic variant, was significantly associated with markers of PH susceptibility and severity (Table 2). The T allele increased PH risk in SCD patients and decreased *RASA3* PBMC expression previously seen in the SCD patients with PH in the UIC cohort.

TABLE 2 rs9525228 is associated with PH susceptibility and severity in SCD.

	β	OR (95% CI)	p
TRV ≥ 2.5 m/s (adult) TRV ≥ 2.6 m/s (PUSH)	0.597	1.81 (1.12–2.95)	0.016
PH	1.92	6.81 (2.03–22.84)	0.0019
Precapillary PH	2.54	12.70 (3.07–52.63)	0.00046
Postcapillary PH	1.33	3.79 (0.62–23.26)	0.15
PVR ≥ 1.5 WU	1.68	5.34 (1.4–20.38)	0.014
PVR ≥ 2.0 WU	1.68	6.97 (1.53–31.71)	0.012
PVR ≥ 3.0 WU	2.46	11.71 (1.61–85.11)	0.015

Note: Using both noninvasive and invasive methods to screen for PH, rs9525228 correlates with markers of precapillary PH. Using a traditional cutoff of PVR < 2 WU for a population with SCD, the median PVR was 1.25 with a 95% CI of 1.00–1.51. Nominal *p* values are reported.

Abbreviations: CI, confidence intervaL; OR, odds ratio; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; SCD, sickle cell disease; TRV, tricuspid regurgitation jet velocity; WU, Wood units.

TABLE 3 Baseline characteristics of PAH Biobank cohort.

	n = 1491
Age	57.5 (14.5)
Sex, % Female	77.3%
% IPAH/HPAH (*n*)	49.0 (731)
% APAH (*n*)	45.5% (678)
% Drug-induced PAH (*n*)	5.5% (82)
mPAP, mmHg	49.6 (14.0)
PCWP, mmHg	10.1 (4.1)
PVR (WU)	9.98 (5.85)

Note: Where applicable, data are presented as mean, (standard deviation).

Abbreviations: APAH, associated pulmonary arterial hypertension; HPAH, hereditary pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; WU, Wood units.

Furthermore, there was a trend toward increased incidence of stroke among patients with the T allele ($p = 0.056$). The SNP was marginally associated with RASA3 expression in PBMC samples ($p = 0.041$), which may be attributed to tissue specificity.

Association of rs9525228 with disease severity in subjects with PAH

Given the common features between SCD-associated PH and PAH we next sought to understand whether rs9525228 contributes to PAH independent of SCD. We, therefore, interrogated the SNP in a cohort of individuals with PAH using data from the PAH Biobank. Baseline characteristics of this cohort are shown in Table 3 (additional ancestry data available in Supporting Information: Figure 1). In this cohort, of 1181 individuals with available information, 232 were on dual therapy and 238 were on triple therapy (including oral or parenteral prostacyclins). An additional 274 patients were on any form of a prostacyclin. In the EA cohort, rs9525228 was associated with higher mPAP ($\beta = 1.52$ per T allele, $p = 0.01$), mean right atrial pressure (mRAP, $\beta = 0.52$ per T allele, $p = 0.04$), and PVR ($\beta = 0.54$ per T allele, $p = 0.04$). There were no significant associations between severity measures and the variant in the AA cohort (Table 4). The MAF varied significantly between individuals of European ancestry (MAF = 0.21) compared with individuals of African ancestry (MAF = 0.036). There were no differences between carriers and noncarriers with functional status as evidenced by 6MWT and NYHA Functional Class. There was a trend toward decreased survival between rs9525228 and individuals from EA ($p = 0.08$) that was not seen among individuals from AA (Table 5).

TABLE 4 rs9525228 is associated with PH severity in PAH according to ancestry.

	European ancestry				African ancestry			
Severity measure	*n*	β	Standard error	*p*	*n*	β	Standard error	*p*
6MWD	843	−15.33	11.75	0.19	139	4.72	38.89	0.9
CI	979	−0.02	0.06	0.75	144	−0.07	0.16	0.69
mPAP	1488	1.52	0.61	0.01	245	1.35	1.88	0.47
mRAP	1458	0.52	0.25	0.04	241	0.43	0.93	0.65
NYHA functional class	1090	−0.02	0.04	0.58	196	0.09	0.12	0.45
PCWP	1451	0.22	0.19	0.25	196	1.01	0.62	0.11
PVR	1424	0.54	0.26	0.04	196	−0.67	1.03	0.52

Abbreviations: 6MWD, 6 min walk distance; CI, cardiac index; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PAH, pulmonary arterial hypertension; PCWP, pulmonary capillary wedge pressure; PH, pulmonary hypertension; PVR, pulmonary vascular resistance.

TABLE 5 Mortality effect of rs9525228 in PAH according to ancestry.

	European ancestry				African ancestry			
	n	Deceased	HR (95% CI)	*p*	*n*	Deceased	HR (95% CI)	*p*
Survival	1490	469	1.15 (0.99–1.33)	0.08	245	53	1.24 (0.62–2.48)	0.55

Abbreviations: CI, confidence interval; HR, hazard ratio; PAH, pulmonary arterial hypertension.

DISCUSSION

This is the first study to associate RASA3 with SCD-associated PH and PAH susceptibility and hemodynamics. Our results suggest that RASA3 may be a novel biomarker and potential candidate gene for pulmonary vascular disease. Individuals with SCD-associated PH had lower PBMC RASA3 expression that correlated with increased mortality. We also found an eQTL within the RASA3 gene, rs9525228, which correlates with markers of PH severity in SCD-associated PH as well as an independent PAH cohort. Among patients with PAH, rs9525228 and EA, there was a trend toward increased mortality. This trend was not seen with patients with PAH and AA.

RASA3 is a GTPase activating protein responsible for repressing Rap1 and Ras activity. RASA3 plays a major role in erythropoiesis and thrombopoiesis through its interactions with Ras, regulating proliferation, survival, differentiation and actin skeleton organization.[40–44] RASA3 also mediates platelet signaling via Rap1.[17] Recently, RASA3 was identified as an important regulator of endothelial cell adhesion, permeability, and vascular lumen formation through its interactions with Rap1. Conditional knockout of RASA3 in endothelial cells demonstrated failure of vascular lumen formation as well as decreased vascular complexity.[20] Germline mutations in RASA3 are embryonic lethal, thought to be due to a failure of angiogenesis and severe bleeding.[22] Given its varied functions, we postulate that RASA3 is likely involved in multiple pathways that may contribute to the development of PH.

This study highlights associations of an eQTL with markers of PH severity among individuals with SCD-associated PH and PAH. Among patients with SCD-associated PH, there was a significant association between RASA3 expression and mortality, which was not seen within the PAH Biobank cohort. We found that in patients with PAH without SCD, there was a significant association between rs9525228 and hemodynamic markers of severity in the EA cohort. We did not observe this difference in the AA cohort of individuals with PAH. This may be due to different tissue types used in the analysis (PBMC data used for SCD-associated PH cohort, and lung tissue for PAH Biobank cohort). However, the lack of replication in the AA population may suggest limitations including underpowered analyses (sample size, MAF difference) or may reflect population-specific differences of SNP contributions. Future investigations can study the latter with larger cohorts. However, this observation may still be generalizable to individuals with SCD-associated PH, as it is well established that individuals with SCD come from genetically diverse backgrounds.[45,46]

In this study, we use both PBMC as well as lung tissue for evaluating eQTL analyses. It is well established that eQTL effects depend on tissue or cell types as well as environment.[35,47,48] Previous studies have suggested that *cis*-eQTLs are less tissue-specific compared with *trans*-eQTLs, and tissues with higher effect sizes are as equally likely to be those tissues with higher or lower expression levels for the gene.[48,49] Other studies have suggested eQTLs from whole blood may commonly share associations between about half of tested traits,[50] suggesting that blood may be an ideal "beginning point" given its accessibility and ease to obtain. This also highlights the need to evaluate gene regulation in disease-relevant tissues.[50] Using single tissue (FDR ≤ 0.05) and multi-tissue analyses (METASOFT, $m = 1.0$), our data suggest that rs9525228 has a strong correlation with lung tissue (Supporting Information: Figure 3).

Another intriguing finding is that rs9525228 is intronic. As genome-wide and whole genome studies become more commonplace to detect variants of interest, it is increasingly common to find these SNPs within introns or intergenic regions.[49,51] It is thought that these areas of the genome may be acting as enhancers, affecting the function of these disease-associated regulatory elements, which may also be extremely tissue-specific.[51] These regions, however, may simply act as a marker of the genetic variant responsible for the effect.[49] One previous study evaluating rs9525228 within the context of depression identified this SNP as having significantly differential binding capabilities at a dexamethasone-responsive element (NR3C1), suggesting that this SNP may play a role in the stress response to PH.[52] Using the ENCODE database, we have also identified rs9525228 at a putative estrogen receptor alpha binding site, suggesting that there may also be sex differences associated with the functionality of this SNP

(Supporting Information: Figure 4). Further work is needed to elucidate the role of rs9525228, particularly within the context of the lung.

There are a number of limitations to our study. Each of our cohorts had relatively small numbers, but we believe that taken together, this improves the strength of our findings. The patients from our SCD cohorts were predominantly Black, whereas our PAH cohort was primarily white, which may limit the generalizability of our findings to other racial groups. Given the diverse underlying pathophysiological mechanisms of SCD-associated PH, a number of individuals from our three SCD cohorts also had a postcapillary PH component, which may also influence our results. Not all individuals within the SCD cohorts underwent RHC, which could potentially over- or underestimate the true prevalence of precapillary PH within these three cohorts. Furthermore, within the PAH cohort, PBMC RASA3 expression was not measured, but will be performed in future studies. Importantly, we did not intend to use the PAH Biobank cohort as a validation cohort, but rather to see whether genetic variation in RASA3 is also noted within a cohort of individuals with PAH specifically. Given that there are few, if any, biomarkers that could be applied in both SCD-associated PH and PAH, we believe that RASA3 remains a provocative target.

We were only able to stratify rs9525228 within the ancestry constraints of European or African ancestry, but the effect of this eQTL on individuals from more varied ancestries remains unknown. Within the PAH Biobank cohort, nearly half of the patients had APAH. Given the diverse etiologies that may cause APAH, this may account for some of the ancestral differences seen and is a topic for future studies. Although we used data from multiple different prospective cohorts, these samples are subject to enrollment bias, so may not be representative of the broader PAH population. Another question that remains unanswered is whether rs9525228 is directly responsible for the effect seen, or rather is in LD with another SNP that may be responsible for clinical effects. Additional studies are ongoing to decipher whether rs9525228 is the culprit or bystander of this effect.

In conclusion, RASA3 expression appears to be differentially regulated in individuals with SCD-associated PH and PAH. Further studies are warranted to determine how RASA3 may be protective against the development of PH within these two cohorts, both in vitro and in vivo. Studies are ongoing regarding the role of RASA3 in pulmonary artery endothelial cell homeostasis and response to noxious stimuli known to contribute to the development of PH, as well as the potential relationship between RASA3 and estrogen. Further studies are also planned to determine how rs9525228 may contribute to a higher mortality within the PAH population.

AUTHOR CONTRIBUTIONS

This study was designed and supervised by Clare C. Prohaska, Robert S. Stearman, Ankit A. Desai, and RFM. Clare C. Prohaska, Robert S. Stearman, Ankit A. Desai, and Roberto F. Machado assembled the figures and wrote the manuscript. Xu Zhang, Tae-Hwi L. Schwantes-An, and Robert S. Stearman performed primarily data analysis. Clare C. Prohaska, Xu Zhang, Robert S. Stearman, Stanley Hooker, Rick A. Kittles, William C. Nichols, Micheala A. Aldred, Ankit A. Desai, VRG, and Roberto F. Machado contributed to study design and data interpretation. All authors reviewed the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors assure that this work is original, has not been previously pushed and has been submitted only to *Pulmonary Circulation*. All sources have been clearly cited and all research ethics guidelines have been adhered to, without data manipulation. All authorship has been accurately represented.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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