RhoJ is differentially expressed in non-small cell lung cancer and associates with patient survival.

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Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States¹. We mined published microarray data^{2,3,4} to identify differentially expressed genes in NSCLC. We found that the gene encoding RhoJ, the ras homolog family member J (RhoJ) was among those whose expression was most quantitatively different in human NSCLC tumors as compared to the lung. RhoJ expression levels were significantly decreased in NSCLC tumors as compared to the lung, and lower expression of RhoJ in patient tumors was significantly associated with worse overall survival. RhoJ may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: RhoJ, NSCLC, non-small cell lung cancer, systems biology of NSCLC, targeted therapeutics in NSCLC.

1 In 2016, lung cancer resulted in the death of 158,000 Americans; 81% of all patients
2 diagnosed with lung cancer will expire within 5 years⁵. Non-small cell lung cancer (NSCLC) is
3 the most common type of lung cancer, diagnosed in 84% of patients with lung cancer, and 76%
4 of all patients with NSCLC will expire within 5 years⁵. The rational development of targeted
5 therapeutics to treat patients with NSCLC can be supported by an enhanced understanding of
6 fundamental transcriptional features of NSCLC tumors. To discover genes associated with
7 NSCLC tumors in an unbiased fashion and at the systems-level, we mined independently
8 published microarray data^{2,3,4} to compare global gene expression profiles of NSCLC tumors to
9 that of the normal lung. We found recurrent and significant differential expression of the gene
10 encoding Rho GTPase J, formally known as the ras homolog family J, RhoJ, in adenocarcinoma
11 tumors from patients with NSCLC, suggesting RhoJ may be important for NSCLC tumor
12 initiation or progression.

13 **Methods**

14 We utilized microarray datasets GSE74706², GSE43458³, GSE33532⁴ and for this
15 differential gene expression analysis of NSCLC tumors in conjunction with GEO2R. GSE74706
16 was generated using Agilent-026652 Whole Human Genome Microarray 4x44K v2 technology;
17 for this analysis, we used $n=18$ control lung tissue and $n=10$ NSCLC tumors, and the analysis
18 was performed using platform GPL13497. GSE43458 was generated using Affymetrix Human
19 Gene 1.0 ST Array technology; for this analysis, we used with $n=30$ control lung tissue and $n=80$
20 NSCLC tumors, and the analysis was performed using platform GPL6244. GSE33532 was

generated using Affymetrix Human Genome U133 Plus 2.0 Array technology; for this analysis, we used $n=20$ control lung tissue and $n=10$ NSCLC tumors, and the analysis was performed using platform GPL570. All tumors utilized for differential gene expression analysis here were of the adenocarcinoma type.

The Benjamini and Hochberg method of p -value adjustment was used for ranking of differential expression but raw p -values were used to assess statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used. A statistical test was performed to evaluate whether RhoJ expression was significantly between normal lung tissue and NSCLC tumors using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses of differential gene expression in NSCLC tumors (Version 8.4.0)(455). For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁷ for correlation of RhoJ mRNA expression levels with overall survival in $n=1144$ non-small cell lung cancer patients.

Results

We harnessed the power of multiple, independently published microarray datasets^{2,3,4} to discover in an unbiased fashion and at the transcriptome-level the most striking gene expression features of NSCLC tumors.

RhoJ is differentially expressed in non-small cell lung cancers.

We found significant differential expression of the gene encoding the ras homology

1 family member J, RhoJ, in NSCLC tumors when compared to the lung² (Table 1). When sorting
2 each of the transcripts measured based on significance of difference in expression of RhoJ
3 between NSCLC tumors and the normal lung, in this dataset, RhoJ ranked 206 out of 34183 total
4 transcripts (Table 1). Differential expression of RhoJ in NSCLC tumors was statistically
5 significant (Table 1; $p=2.43E-11$).
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8 We queried a second microarray dataset³ to determine if we could validate differential
9 expression of RhoJ in non-small cell lung cancers (Table 2). We found significant differential
10 expression of RhoJ in NSCLC tumors of the adenocarcinoma type when compared to the normal
11 lung (Table 2). When sorting each of the transcripts measured based on significance of
12 difference in expression of RhoJ between NSCLC tumors and the normal lung, RhoJ ranked 228
13 of 33252 total transcripts (Table 2). Differential expression of RhoJ in NSCLC tumors was
14 statistically significant (Table 2; $p=2.38E-20$).
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17 Analysis of a third microarray dataset⁴ again revealed significant differential expression
18 of RhoJ in NSCLC tumors of the adenocarcinoma type. When sorting each of the transcripts
19 measured based on significance of difference in expression of RhoJ between NSCLC tumors and
20 the normal lung, RhoJ ranked 436 out of 25906 total transcripts (Table 3). Differential
21 expression of RhoJ in NSCLC tumors was statistically significant (Table 3; $p=3.45E-10$).
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25 RhoJ is expressed at significantly lower levels in NSCLC tumors as compared to the lung.
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27 We obtained exact mRNA levels for RhoJ from NSCLC tumors and from the lung to
28 directly compare RhoJ expression between tumor and control lung tissue and assess for

1 statistical significance. RhoJ was expressed at significantly lower levels in NSCLC tumors as
2 opposed to normal lung in each dataset queried, respectively (Figure 1: $p<0.0001$, Figure 2:
3 $p<0.0001$). We calculated a mean fold change of 0.841 ± 0.075 in RhoJ expression when
4 comparing NSCLC tumors to the lung (Table 2).
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6 RhoJ expression in NSCLC tumors correlates with overall survival.

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8 We performed Kaplan-Meier survival analysis using RhoJ mRNA expression in NSCLC
9 tumors coupled with paired overall survival data from each patient, in 1144 NSCLC patients in
10 total, to determine whether RhoJ tumor expression was correlated with survival outcomes in
11 NSCLC. We found that patients whose tumors expressed lower levels of RhoJ possessed
12 significantly shorter overall survival than patients with high tumor expression of RhoJ (Figure 3).
13 Median overall survival (OS) of patients in the low expression cohort was 63 months, while
14 median OS in patients in the high RhoJ expression cohort was 103 months (Table 4); this
15 difference in median OS based on RhoJ tumor expression in NSCLC was statistically significant
16 (Figure 3; logrank p -value: $5.7e-6$; hazard ratio: 0.65 (0.54 - 0.78); false discovery rate=0.01).
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19 Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed
20 differential expression of transcripts encoded by the RhoJ gene as among the most significant
21 transcriptional features of NSCLC tumors, and RhoJ expression was significantly correlated with
22 patient outcomes, as patients with lower tumor expression of RhoJ possessed significantly worse
23 overall survival.
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Discussion

The Rho guanosine triphosphatases (RhoGTPase) RhoJ, also known as TCL, bears 78% homology to CDC42, and 85% homology to TC10 (RhoQ), and was thus named TC10-like, or TCL⁶. GTP-bound RhoJ can interact with the Cdc42/Rac-binding domain, found in proteins including WASP and PAK⁶. RhoJ can induce morphological changes, which can be antagonized by dominant negative Rac1 and Cdc42 mutants, indicating that that pathways that these three proteins influence intersect with one another⁶. RhoJ can localize both to the plasma membrane and to the early endosomes⁷. In the endocytic pathway, RhoJ functions in entry of clathrin-coated vesicle cargo such as transferrin into the early endocytic pathway⁷. RhoJ is thought to possess specific expression in endothelial cells⁸. RhoJ can also function in regulation of cellular motility behaviors through incorporation into focal adhesions through interactions with the GIT-PIX complex through the Spa homology domain of GIT1 to regulate focal adhesion disassembly, and together, GIT1, GIT2, β -PIX (ARHGEF7) and RhoJ can support formation of endothelial tubes⁹. Subcutaneous implantation of Lewis lung carcinoma cells into RhoJ knockout mice results in significant reduction in tumor mass and number of vessels per field, suggesting that RhoJ function in cells and tissues outside of the tumor can influence tumor dynamics including vessel biology⁹. The $\alpha 5 \beta 1$ integrin can reside within RhoJ+ endosomes of endothelial cells, and RhoJ can antagonize Cdc42 function by competing with Cdc42 for PAK3 binding to repress fibronectin fibrillogenesis¹⁰. RhoJ expression can be induced by vascular endothelial growth factor (VEGF), and RhoJ expression can be found in vasculature of both normal and cancerous

1 tissues and during development¹¹. Other functions of RhoJ is in negative regulation of
2 actomyosin contractility as well as stress fiber formation¹¹. RhoJ can influence migration of
3 endothelial cells, integrating repulsive and attractant guidance cues by VEGF and semaphorin
4 3E, respectively: RhoJ can interact with PlexinD1, and Sema3A stimulation results in
5 disassembly of this interaction and promotion of cell contractility (repulsion), while VEGF
6 stimulation results in formation of a VEGFR2, PlexinD1, and neuropilin-1 for which RhoJ is
7 required and subsequent endothelial cell migration (attraction)¹².

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11 RhoJ has important roles in cancer cells and its perturbed expression has been described
12 in multiple human cancers. In mouse models of solid tumors including the Lewis lung
13 carcinoma (LLC) and B16/F10 melanoma model, RhoJ has relatively high expression in tumor
14 endothelial cells and in human patients with colorectal cancer, overall survival is significantly
15 higher in patients with low expression of RhoJ, different from our analysis of published
16 microarray data¹³. LLC tumors in RhoJ-null mice manifest disruption of tumor vessels and
17 decreased vascular density¹³. Lymph node metastasis in the B16/F10 melanoma model was
18 significantly decreased, by 47%¹³. In gastric cancer, higher expression of RhoJ correlates with
19 decreased progression-free and overall survival and YCC-16 gastric cancer cells ectopically
20 expressing RhoJ display increased migratory and invasive capacity *in vitro*¹⁴. RhoJ is over-
21 expressed in the tumors of patients with glioblastoma and its expression associates with patient
22 survival¹⁵. We identified one recent study describing a role for RhoJ in NSCLC, finding,
23 consistent with our analysis, decreased expression of RhoJ in NSCLC tumors¹⁶.

1 We found, by mining multiple independently published microarray datasets, that RhoJ
2 was among the genes most differentially expressed in the primary tumors of patients with non-
3 small cell lung cancer; RhoJ was expressed at significantly lower levels in NSCLC tumors as
4 compared to the lung, and decreased expression of RhoJ was significantly associated with worse
5 overall survival in NSCLC patients. RhoJ may be of relevance to the biology of tumor initiation
6 or progression in patients with NSCLC of the adenocarcinoma type, the most common type of
7 the leading cause of cancer death in the United States and worldwide.
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Rank	ID	p-value	t	B	Gene
206	A_24_P82032	2.43E-11	-1.05E+01	15.9632794	RHOJ

Table 1: RhoJ is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, fold change of RhoJ expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
228	7974902	2.38E-20	-11.373144	35.757468	0.841 ± 0.075	RHOJ	ras homolog family member J

Table 2: RhoJ is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
436	3.45E-10	-10.716624	17.2906586	-1.4040839	RHOJ	ras homolog family member J

Table 3: RhoJ is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, fold change of RhoJ expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Figure 1: RhoJ is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of RhoJ is graphically represented in the lung (left) and in NSCLC tumors of the adenocarcinoma type (right) with mean mRNA expression values marked and the result of a statistical test evaluating significance of difference in RhoJ expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: RhoJ transcript is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of RhoJ is graphically represented in the lung (left) and in NSCLC tumors of the adenocarcinoma type (right) with mean mRNA expression values marked and the result of a statistical test evaluating significance of difference in RhoJ expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: RhoJ expression in NSCLC tumors significantly correlates with overall survival.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=1144$ total patients stratified into two groups, based on low or high expression of RhoJ in patient tumors. The log rank p -value denoting statistical significance of difference in overall survival when comparing the two groups, as well as hazard ratio for this comparison is listed above. Listed below is the number of patients at risk (number of patients alive) per interval, after stratification based on RhoJ expression; in the first interval, number at risk is number of patients alive; in each subsequent interval, number at risk is the number at risk less those who have expired or are censored.

Low expression cohort (months)	High expression cohort (months)
63	103

Table 4: RhoJ expression in NSCLC tumors significantly correlates with patient survival.

The median overall survival of $n=1144$ NSCLC patients based on stratification into low or high expression of RhoJ in tumors is listed in this chart.