The roundabout guidance receptor 4, ROBO4, 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 ROBO4 was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. ROBO4 expression was significantly decreased in NSCLC tumors as compared to the lung, and lower expression of ROBO4 in patient tumors was significantly associated with worse overall survival. ROBO4 may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: ROBO4, 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 ROBO4 in
10 adenocarcinoma tumors from patients with NSCLC, suggesting ROBO4 may be important for
11 NSCLC tumor initiation or progression.

12 **Methods**

13 We utilized microarray datasets GSE33532³, GSE74706⁴ and GSE43458⁵ and this
14 differential gene expression analysis of NSCLC tumors in conjunction with GEO2R. GSE33532
15 was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology; for this
16 analysis, we used $n=20$ control lung tissue and $n=10$ NSCLC tumors, and the analysis was
17 performed using platform GPL570. GSE74706 was generated using Agilent-026652 Whole
18 Human Genome Microarray 4x44K v2 technology; for this analysis, we used $n=18$ control lung
19 tissue and $n=10$ NSCLC tumors, and the analysis was performed using platform GPL13497.
20 GSE43458 was generated using Affymetrix Human Gene 1.0 ST Array technology; for this

analysis, we used with $n=30$ control lung tissue and $n=80$ NSCLC tumors, and the analysis was performed using platform GPL6244. 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 ROBO4 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 ROBO4 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.

ROBO4 is differentially expressed in non-small cell lung cancers.

We found significant differential expression of the roundabout guidance receptor 4, ROBO4 in NSCLC tumors when compared to the lung² (Table 1). When sorting each of the

transcripts measured based on significance of difference in expression of ROBO4 between NSCLC tumors and the normal lung, ROBO4 ranked 69 out of 25906 total transcripts (Table 1). Differential expression of ROBO4 in NSCLC tumors was statistically significant (Table 1; $p=1.33E-15$).

We queried a second microarray dataset³, to determine if we could validate differential expression of ROBO4 in non-small cell lung cancers. We again found significant differential expression of ROBO4 in NSCLC tumors of the adenocarcinoma type when compared to the normal lung (Table 2). When sorting each of the transcripts measured based on significance of difference in expression of ROBO4 between NSCLC tumors and the normal lung, in this dataset, ROBO4 ranked 24 out of 34183 total transcripts (Table 2). Differential expression of ROBO4 in NSCLC tumors was statistically significant (Table 2; $p=4.4E-14$).

Analysis of a third microarray dataset⁴ revealed significant differential expression of ROBO4 in NSCLC tumors of the adenocarcinoma type (Table 3). When sorting each of the transcripts measured based on significance of difference in expression of ROBO4 between NSCLC tumors and the normal lung, ROBO4 ranked 57 out of 33252 total transcripts (Table 3). Differential expression of ROBO4 in NSCLC tumors was statistically significant (Table 3; $p=6.17E-26$).

ROBO4 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for ROBO4 from NSCLC tumors and from the lung to directly compare ROBO4 expression between tumor and control lung tissue and assess for

1 statistical significance. ROBO4 was expressed at significantly lower levels in NSCLC tumors as
2 compared to the normal lung in each dataset queried (Figure 1: $p<0.0001$, Figure 2: $p<0.0001$,
3 Figure 3: $p<0.0001$). We calculated a mean fold change of 0.7386 ± 0.0562 (Table 1) and
4 0.8635 ± 0.0481 (Table 3) in ROBO4 expression when comparing NSCLC tumors to the lung.
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7 ROBO4 expression in NSCLC tumors correlates with overall survival.

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9 We performed Kaplan-Meier survival analysis using ROBO4 mRNA expression in
10 NSCLC tumors coupled with paired overall survival data from each patient, in 1144 NSCLC
11 patients in total, to determine whether ROBO4 tumor expression was correlated with survival
12 outcomes in NSCLC. We found that patients whose tumors expressed lower levels of ROBO4
13 possessed significantly shorter overall survival than patients with high tumor expression of
14 ROBO4 (Figure 4). Median overall survival (OS) of patients in the low expression cohort was
15 44.83 months, while median OS in patients in the high ROBO4 expression cohort was 105
16 months (Table 4); this difference in median OS based on ROBO4 tumor expression in NSCLC
17 was statistically significant (Figure 4; logrank p -value: $2.5e-11$; hazard ratio: $0.57 (0.48-0.68)$).
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21 Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed
22 differential expression of ROBO4 as among the most significant transcriptional features of
23 NSCLC tumors, and ROBO4 expression was significantly correlated with patient outcomes, as
24 patients with lower tumor expression of ROBO4 possessed significantly worse overall survival.
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Discussion

Migration of growth cones in the nervous system is controlled by a number of different families of genes, including Slits, which are ligands for receptors of the roundabout, or Robo receptor gene family⁶. Formation and patterning of the vasculature, such as that which occurs during sprouting of blood vessels, can also be influenced by guidance cues. The Robo receptor Robo4 is expressed in vascular endothelium during mouse development⁷. Two interaction partners of Robo4 are Slit and Mena⁷. In primary endothelial cells, Robo4 is the only Robo family member expressed. Robo4 was identified based on the fact that mice deficient in Alk1 (also known as ACVRL1), which manifest significant defects in vascular development, up-regulate Robo4 mRNA 5-fold⁷. Robo4 has important functions in angiogenesis and in patterning of the vasculature⁷. Both murine and human Robo4 contain 2 immunoglobulin domains as well as two fibronectin domains, which comprise the extracellular ligand-binding portion of Robo4; while other Robos contain 4 conserved cytoplasmic domains CC0, CC1, CC2 and CC3, Robo4 only possesses CC domains CC0 and CC2⁷.

In murine development, expression of Robo4 can be detected as early as embryonic day 7 (E7) and in the central vessels, the dorsal aorta, and intersomitic vessels at E9.0⁷. In adult mice, Robo4 is expressed most abundantly in the heart, but with expression also observable in the liver, lungs, kidney and less so in the brain⁷. In Robo4-expressing HEK cells, Robo4-Slit interaction results in inhibition of cell migration, as well as in primary human endothelial cells that express Robo4⁷. Robo4 can also function in maintenance of vascular integrity⁸. Slit2 can activate

Robo4, leading to inhibition of migration induced by vascular endothelial growth factor 165 (VEGF-165) as well as vascular leaking caused by VEGF-165 through inhibition of Src kinase activation⁸. By interacting with the paxillin molecule, Robo4 can block activation of the Arf6 GTPase, as well as Rac by recruiting Arf GTPase activating proteins (Arf GAPs) such as GIT1⁹. In this manner, Robo4 is able to regulate cellular protrusion associated with the process of neovascularization. Another interaction partner of Robo4 is the Netrin receptor Unc5B; this interaction supports vascular integrity through inhibition of endothelial cell VEGF signaling¹⁰. In a mouse subcutaneous sponge model, soluble Robo4 could inhibit angiogenesis induced by injection of the basic fibroblast growth factor (bFGF)¹¹. Soluble Robo4 can also inhibit the migration and proliferation of human umbilical vein endothelial cells (HUVEC)¹¹. However, contrary to other findings, one report argued that Robo4 could not interact with any Slit family proteins¹¹. In a model of retinopathy induced by oxygen, deletion of Robo4 supports permeability and revascularization¹². Robo4 functioned in oxygen-induced retinopathy by antagonizing tyrosine phosphorylation at residue 949 of the vascular endothelial growth factor receptor 2 (VEGFR2) via engagement of Unc5B¹². In the context of angiogenic stimulus, ablation of Robo4 in the endothelium can phenocopy increased vessel angiogenesis resulting from loss of Slit in the mammary stroma, through activation of VEGFR2 via Src and focal adhesion kinase signaling, and in the breast, expression of Slit in the stroma could impair growth of blood vessels by down-regulating VEGFR2 signaling through ROBO4 in the endothelium¹³. In pulmonary microvascular endothelial cells infected with Andes or Hantaan viruses,

1 interactions between Robo4 and Slit2 can inhibit permeability and disassembly of adherens
2 junctions¹⁴. Thus, altogether, Robo4 has influence over migration of endothelial cells and
3 vascularization and can support vascular integrity in the context of VEGF signaling.
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5 Dysregulated expression of Robo4 has been implicated in human cancer. One study
6 documented increased expression of Robo4 in colorectal cancers attributable to over-expression
7 of Robo4 on endothelial cells of tumor blood vessels¹⁵. Contrary to what we found across
8 multiple independent datasets, increased rather than decreased expression of Robo4 was
9 described in resected tumor tissue from 63 NSCLC patients as compared to adjacent non-
10 cancerous tissue¹⁶. However, in agreement with our findings here, the same study found that
11 higher tumor expression of Robo4 correlated with increased overall survival in NSCLC
12 patients¹⁶.
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16 We found, by mining multiple independently published microarray datasets, that the
17 roundabout guidance receptor Robo4 was among the genes most differentially expressed in the
18 the primary tumors of patients with non-small cell lung cancer; Robo4 was expressed at
19 significantly lower levels in NSCLC tumors as compared to the lung, and decreased expression
20 of Robo4 was significantly associated with worse overall survival in NSCLC patients. Robo4
21 may be of relevance to the biology of tumor initiation or progression in patients with NSCLC of
22 the adenocarcinoma type, the most common type of the leading cause of cancer death in the
23 United States and worldwide.
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Rank	ID	p-value	t	B	FC	Gene	Gene name
69	226028_at	1.33E-15	-14.777196	25.5988028	0.7386 ± 0.0562	ROBO4	roundabout guidance receptor 4

Table 1: ROBO4 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 ROBO4 expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	Gene
24	A_23_P344421	4.4E-14	-1.36E+01	22.1314778	ROBO4

Table 2: ROBO4 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 and gene are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
57	7952453	6.17E-26	-13.838633	48.445259	0.8635 ± 0.0481	ROBO4	roundabout guidance receptor 4

Table 3: ROBO4 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 ROBO4 expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Figure 1: ROBO4 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ROBO4 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 ROBO4 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: ROBO4 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ROBO4 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 ROBO4 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: ROBO4 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ROBO4 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 ROBO4 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 4: ROBO4 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 ROBO4 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 ROBO4 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)
44.83	105

Table 4: ROBO4 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 ROBO4 in tumors is listed in this chart.