The Activin-A receptor-like protein 1, ACVRL1 is differentially expressed in non-small cell lung cancer.

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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^{2,3,4,5} to identify differentially expressed genes in NSCLC. We found that the gene encoding the Activin-A receptor-like protein 1, ACVRL1, was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. ACVRL1 may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: ACVRL1, 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 years⁶. 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 years⁶. 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^{2,3,4,5} 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
10 Activin-A receptor-like protein 1, ACVRL1, in adenocarcinoma tumors from patients with
11 NSCLC, suggesting ACVRL1 may be important for NSCLC tumor initiation or progression.
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16 **Methods**

17 We utilized microarray datasets GSE74706², GSE43458³, GSE33532⁴ and GSE62113⁵
18 for this differential gene expression analysis of NSCLC tumors in conjunction with GEO2R.
19 GSE74706 was generated using Agilent-026652 Whole Human Genome Microarray 4x44K v2
20 technology; for this analysis, we used $n=18$ control lung tissue and $n=10$ NSCLC tumors, and the
21 analysis was performed using platform GPL13497. GSE43458 was generated using Affymetrix
22 Human Gene 1.0 ST Array technology; for this analysis, we used with $n=30$ control lung tissue
23 and $n=80$ NSCLC tumors, and the analysis was performed using platform GPL6244. GSE33532
24 was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology; for this
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analysis, we used $n=20$ control lung tissue and $n=10$ NSCLC tumors, and the analysis was performed using platform GPL570. GSE62113 was generated using Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip technology; for this analysis, we used $n=9$ control lung tissue and $n=7$ NSCLC tumors, and the analysis was performed using platform GPL14951. 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 ACVRL1 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 (Version 8.4.0)(455).

Results

We harnessed the power of multiple, independently published microarray datasets^{2,3,4,5} to discover in an unbiased fashion and at the transcriptome-level the most striking gene expression features of NSCLC tumors.

ACVRL1 is differentially expressed in non-small cell lung cancers.

We found significant differential expression of the gene encoding the Activin-A receptor-like protein 1, ACVRL1, in NSCLC tumors when compared to the lung² (Table 1). When

1 sorting each of the transcripts measured based on significance of difference in expression of
2 ACVRL1 between NSCLC tumors and the normal lung, ACVRL1 ranked 124 out of 34183 total
3 transcripts (Table 1). Differential expression of ACVRL1 in NSCLC tumors was statistically
4 significant (Table 1; $p=3.38E-12$).
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6 Analysis of a second microarray dataset³ again revealed significant differential
7 expression of ACVRL1 in NSCLC tumors of the adenocarcinoma type (Table 2). When sorting
8 each of the transcripts measured based on significance of difference in expression of ACVRL1
9 between NSCLC tumors and the normal lung, ACVRL1 ranked 24 out of 33252 total transcripts
10 (Table 2). Differential expression of ACVRL1 in NSCLC tumors was statistically significant
11 (Table 2; $p=3.87E-29$).
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14 We queried a third microarray dataset⁴, again finding significant differential expression of
15 ACVRL1 in non-small cell lung cancers of the adenocarcinoma type when compared to the
16 normal lung. When sorting each of the transcripts measured based on significance of difference
17 in expression of ACVRL1 between NSCLC tumors and the normal lung, in this dataset,
18 ACVRL1 ranked 127 out of 25906 total transcripts (Table 3). Differential expression of
19 ACVRL1 in NSCLC tumors was statistically significant (Table 3; $p=2.9E-14$).
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23 In a fourth dataset⁵, we again observed significant differential expression of ACVRL1 in
24 human NSCLC tumors when compared to normal lung. When sorting each gene by significance
25 of difference in expression between NSCLC tumors and the normal lung, ACVRL1 ranked 122
26 out of 29376 total transcripts (Table 4). Differential expression of ACVRL1 in this fourth dataset
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was again statistically significant (Table 4; $p=8.48E-06$).

ACVRL1 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for ACVRL1 from NSCLC tumors and from the lung to directly compare ACVRL1 expression between tumor and control lung tissue and assess for statistical significance. ACVRL1 was expressed at significantly lower levels in NSCLC tumors as compared to the normal lung in each of the datasets queried (Figure 1: $p<0.0001$; Figure 2: $p<0.0001$; Figure 3: $p<0.0001$; and Figure 4: $p=0.0002$). We calculated a fold change of 0.8432 ± 0.0524 (Table 2), 0.7677 ± 0.0411 (Table 3) and 0.8305 ± 0.0673 (Table 4) in ACVRL1 expression when comparing NSCLC tumors to the lung.

Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed differential expression of ACVRL1 as among the most significant transcriptional features of NSCLC tumors.

Discussion

The major function of ACVRL1, also known as the activin receptor-like kinase (ALK1), appears to be in the transduction of transforming growth factor beta receptor (TGF β R) signals in the endothelium⁷. There are three ligands for ACVRL1. In conjunction with TGFBR-II, ACVRL1 can transduce signals following ligation with the ligands TGF- β 1 and TGF- β 3⁸. In conjunction with the type IIA Activin Receptor or the type II bone morphogenetic protein (BMP)

receptor, ACVRL1 can transduce signals following interactions with BMP-9, with phosphorylation of Smad1, Smad5 and Smad8 in microvascular endothelial cells resulting from BMP-9-ACVRL1 ligation⁹. ACVRL1-knockout mice are embryonic lethal; at embryonic day 9.5 (E9.5), mature blood vessels are completely absent from the yolk sac of ACVRL1 ^{-/-} embryos¹⁰. While vasculogenesis is intact in ACVRL1^{-/-} embryos, angiogenesis is defective; ACVRL1^{-/-} embryos are resorbed by E11.5, and between E9.5 and E10.5 ACVRL1^{-/-} embryos feature vascular defects characterized by capillary fusion and dilated yolk sac vasculature¹⁰. In addition to transducing TGF- β 1 signals, with phosphorylation of Smad1 and Smad5 following ligation of ACVRL1 by TGF- β 1 in human umbilical vein endothelial cells, ACVRL1 can inhibit transduction of signals following ligation of TGF β R1/ALK5 by TGF- β 1¹⁰. The zebrafish mutant violet beauregarde features abnormal circulation at 2 days post-fertilization, with failure of blood cells to perfuse the trunk and tail. This defect appears to be a result of an increase in endothelial cell number in specific cranial vessels¹¹. Conditional knockout of ACVRL1 in neonatal mice using a tamoxifen-inducible Cdh5-Cre revealed pulmonary hemorrhage from the distal small capillaries, accompanied by respiratory distress and mortality¹². In the neonatal retina, hyper branching of the retinal plexus was observed, with enlarged veins, and an increase in tip cell numbers in the central plexus¹². While neonatal mice with inducible knockout of ACVRL1 suffered from retinal hemorrhage, inducible knockout of ACVRL1 in adult mice resulted in caecal hemorrhage as a result of fragility of the microvessels in the villi of the caecum¹².

Hereditary hemorrhagic telangiectasia (HHT), also known as Rendu-Osler-Weber (ROW)

1 syndrome or Osler-Rendu-Weber (ORW) syndrome, is an inherited vascular dysplasia featuring
2 vascular defects in the skin, brain lungs and gastrointestinal tract¹³: type II HHT (HHT2) is
3 caused by mutations in ACVRL1¹⁴. Arteriovenous malformations can be found in the lungs of 5
4 to 15% of patients with HHT - direct connections between the pulmonary artery and pulmonary
5 vein resulting from a thin-walled aneurysm¹³. Thus, ACVRL1 mutations can result in defects in
6 pulmonary vasculature in humans.
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10 Perturbed expression of ACVRL1 has been reported in human cancer. A study using
11 computational analysis to investigate genes co-expressed with ACVRL1 in a variety of tumors
12 found that the expression of 8 genes was associated with ACVRL1 expression, including
13 PECAM1 and ROBO4, as well as CLEC14A¹⁵. This gene set was enriched for biological
14 processes including angiogenesis and blood vessel development. Stimulation of murine MS1
15 endothelial cells with BMP9 resulted in induction of CLEC14A expression while TGF- β 1
16 stimulation resulted in repression of CLEC14A expression¹⁵. Chromatin immunoprecipitation
17 coupled with RNA-sequencing (ChIP-seq) in HUVEC stimulated with either BMP6 or BMP9
18 revealed Smad1/5 occupancy at the Clec14A promoter¹⁵. Together, the data suggested that
19 ACVRL1 was co-expressed in cancers along with a number of genes involved in angiogenesis or
20 endothelial cell function, and that in some cases, (i.e., Clec14A), signal transduction downstream
21 of ACVRL1 was linked with this expression¹⁵. A study of 169 tumors from patients with head
22 and neck cancer demonstrated that high levels of ACVRL1 expression were significantly
23 associated with worse patient outcomes, including advanced TNM tumor stage, extracapsular
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spread of lymph node metastasis and worse 5-year overall survival¹⁶. Moreover, in the TRIBE study of patients with metastatic colorectal cancer, higher expression of ACVRL1 was associated with enhanced patient outcomes including overall survival in patients receiving FOLFOXIRI-bevacizumab or FOLFIRI-bevacizumab¹⁶. However, consistent with what we found in NSCLC tumors across multiple datasets, decreased expression of ACVRL1 has been documented in NSCLC of the adenocarcinoma type¹⁵. A fusion protein generated from the extracellular domain of ACVRL1 fused to IgG₁-Fc, RAP-041, could inhibit BMP9-induced transcriptional activation in C2C12 cells, decrease endothelial cell cord length by 42% in HUVEC grown on Matrigel-coated plates, and reduce angiogenic sprouting induced by vascular endothelial growth factor (VEGF) in a 3-dimensional endothelial cell spheroid sprouting assay. Use of the RIP1-Tag2 pancreatic beta-cell cancer mouse model to study angiogenesis in multi-step carcinogenesis revealed that RAP-041 administration *in vivo* resulted in significant reduction of tumor burden both early in tumor development and later in mice with bulky tumors. Vascular density and perfusion were also significantly decreased in tumors from mice treated with RAP-041. Thus, in at least one mouse model of cancer, a therapeutic targeting ACVRL1 has demonstrated efficacy in inhibiting tumor growth and angiogenic processes.

We found that the gene encoding the Activin-A receptor-like protein 1, ACVRL1, was among the genes whose expression was most significantly different in NSCLC tumors of the adenocarcinoma type when compared to normal lung tissue, with significantly decreased tumor expression of ACVRL1 in NSCLC. ACVRL1 may be of relevance to the initiation or progression of NSCLC tumors in humans.

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Rank	ID	p-value	t	B	Gene
124	A_24_P945113	3.38E-12	-1.14E+01	17.9020011	ACVRL1

Table 1: ACVRL1 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
24	7955562	3.87E-29	-15.30447	55.709536	0.8432 ± 0.0524	ACVRL1	activin A receptor like type 1

Table 2: ACVRL1 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 ACVRL1 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
127	226950_at	2.9E-14	-13.177706	22.5555515	0.7677 ± 0.0411	ACVRL1	activin A receptor like type 1

Table 3: ACVRL1 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 ACVRL1 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
122	ILMN_1807493	8.48E-06	-6.5070767	3.876777	0.8305 ± 0.0673	ACVRL1

Table 4: ACVRL1 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 ACVRL1 expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Figure 1: ACVRL1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ACVRL1 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 ACVRL1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: ACVRL1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ACVRL1 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 ACVRL1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: ACVRL1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ACVRL1 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 ACVRL1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 4: ACVRL1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ACVRL1 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 ACVRL1 expression between NSCLC tumors and the lung, a *p*-value, listed above.