The natriuretic peptide receptor, NPR1 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 NPR1 was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. NPR1 expression was significantly decreased in NSCLC tumors as compared to the lung, and lower expression of NPR1 in patient tumors was significantly associated with worse overall survival. NPR1 may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: NPR1, 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 NPR1 in
10 adenocarcinoma tumors from patients with NSCLC, suggesting NPR1 may be important for
11 NSCLC tumor initiation or progression.

12 **Methods**

13 We utilized microarray datasets GSE33532², GSE43458³ and GSE74706⁴ for 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. GSE43458 was generated using Affymetrix Human Gene
18 1.0 ST Array technology; for this analysis, we used with $n=30$ control lung tissue and $n=80$
19 NSCLC tumors, and the analysis was performed using platform GPL6244. GSE74706 was
20 generated using Agilent-026652 Whole Human Genome Microarray 4x44K v2 technology; for

1 this analysis, we used $n=18$ control lung tissue and $n=10$ NSCLC tumors, and the analysis was
2 performed using platform GPL13497. All tumors utilized for differential gene expression
3 analysis here were of the adenocarcinoma type.

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5 The Benjamini and Hochberg method of p -value adjustment was used for ranking of
6 differential expression but raw p -values were used to assess statistical significance of global
7 differential expression. Log-transformation of data was auto-detected, and the NCBI
8 generated category of platform annotation was used. A statistical test was performed to evaluate
9 whether NPR1 expression was significantly between normal lung tissue and NSCLC tumors
10 using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical
11 analyses of differential gene expression in NSCLC tumors (Version 8.4.0)(455). For Kaplan-
12 Meier survival analysis, we used the Kaplan-Meier plotter online tool⁶ for correlation of NPR1
13 mRNA expression levels with overall survival in $n=1925$ non-small cell lung cancer patients.
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19 **Results**

20 We harnessed the power of multiple, independently published microarray datasets^{2,3,4} to
21 discover in an unbiased fashion and at the transcriptome-level the most striking gene expression
22 features of NSCLC tumors.
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25 NPR1 is differentially expressed in non-small cell lung cancers.

26 We found significant differential expression of the gene encoding the natriuretic peptide
27 receptor, NPR1, in NSCLC tumors when compared to the lung² (Table 1). When sorting each of
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the transcripts measured based on significance of difference in expression of NPR1 between NSCLC tumors and the normal lung, NPR1 ranked 40 out of 25906 total transcripts (Table 1). Differential expression of NPR1 in NSCLC tumors was statistically significant (Table 1; $p=1.95E-16$).

We queried a second microarray dataset³ to determine if we could validate differential expression of NPR1 in non-small cell lung cancers (Table 2). We again found significant differential expression of NPR1 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 NPR1 between NSCLC tumors and the normal lung, NPR1 ranked 187 out of 33252 total transcripts (Table 2). Differential expression of NPR1 in NSCLC tumors was statistically significant (Table 2; $p=1.81E-21$).

Analysis of a third microarray dataset⁴ revealed significant differential expression of NPR1 in NSCLC tumors of the adenocarcinoma type. When sorting each of the transcripts measured based on significance of difference in expression of NPR1 between NSCLC tumors and the normal lung, in this dataset, NPR1 ranked 404 out of 34183 total transcripts (Table 3). Differential expression of NPR1 in NSCLC tumors was statistically significant (Table 3; $p=4.69E-10$).

NPR1 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for NPR1 from NSCLC tumors and from the lung to directly compare NPR1 expression between tumor and control lung tissue and assess for

1 statistical significance. NPR1 was expressed at significantly lower levels in NSCLC tumors as
2 compared to normal lung in each dataset queried (Figure 1: $p < 0.0001$, Figure 2: $p < 0.0001$). We
3 calculated a mean fold change of 0.7497 ± 0.0446 and 0.8454 ± 0.0698 in NPR1 expression
4 when comparing NSCLC tumors to the lung (Table 1 and Table 2, respectively).

7 NPR1 expression in NSCLC tumors correlates with overall survival.

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9 We performed Kaplan-Meier survival analysis using NPR1 mRNA expression in NSCLC
10 tumors coupled with paired overall survival data from each patient, in 1925 NSCLC patients in
11 total, to determine whether NPR1 tumor expression was correlated with survival outcomes in
12 NSCLC. We found that patients whose tumors expressed lower levels of NPR1 possessed
13 significantly shorter overall survival than patients with high tumor expression of NPR1 (Figure
14 4). Median overall survival (OS) of patients in the low expression cohort was 52 months, while
15 median OS in patients in the high NPR1 expression cohort was 104 months (Table 4); this
16 difference in median OS based on NPR1 tumor expression in NSCLC was statistically
17 significant (Figure 4; logrank p -value: $3.4e-13$; hazard ratio: 0.59 (0.51 - 0.68); false discovery
18 rate=0.01).

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23 Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed
24 differential expression of NPR1 as among the most significant transcriptional features of NSCLC
25 tumors, and NPR1 expression was significantly correlated with patient outcomes, as patients
26 with lower tumor expression of NPR1 possessed significantly worse overall survival.
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Discussion

Natriuretic peptide receptor, also known as guanylate cyclase A (GC-A), is a receptor bound on the cell surface of the plasma membrane that recognizes the ligand atrial natriuretic peptide receptor (ANP)⁷. The membrane-bound but not soluble form of GC-A/NPR1 is activated following binding of ANP to the cell surface⁸. The result of these events at the cell surface is the production of cyclic GMP. Mice deficient in NPR1 manifest significant elevation in blood pressure and their hearts feature interstitial fibrosis and are hypertrophic⁹. All NPR1-null male mice experience fatality before 6 months of age due to sudden death as a result of congestive heart failure or aortic dissection⁹. Another ligand for NPR1 is the brain natriuretic peptide, also known as neuropeptide NPPB, involved in the itch response, and small molecule inhibitors of NPR1 inhibit itching behavior in a mouse model of acute itch¹⁰. Investigators studying the effect of the most classically defined ligand of NPR1, atrial natriuretic peptide receptor on cancer recurrence after surgery for lung cancer found that 2-year relapse-free survival was significantly higher in patients treated with ANP¹¹. In mouse models of pulmonary metastasis in conjunction with lipopolysaccharide (LPS) treatment which mimics surgical stress, pre-treatment with ANP significantly reduced degree of pulmonary metastasis in the A549 non-small cell lung cancer model and in the B16/F10 melanoma mouse model¹¹. Mechanistically, ANP treatment resulted in decreased expression of E-selectin, impairing adhesion of A549 and H460 cancer cells to vascular endothelium induced by inflammation associated with LPS administration¹¹. Finally, in NPR1-deficient mice, messenger RNA and protein for the Toll-like receptor (TLR) 2 and TLR4

1 in the kidneys were significantly increased, while numbers of Foxp3+ CD4+ CD25+ regulatory
2 T-cells in circulation were significantly decreased¹². Levels of interleukin (IL)-2, interferon
3 gamma, tumor necrosis factor alpha, and IL-17 were all significantly increased in the kidneys in
4 Npr1-null mice, while Th2 cytokines IL-4, IL-5, IL-10 and IL-13 were all significantly
5 decreased¹². NPR1 locus was methylated in 42.5% of gastric cancer in a survey of 120 patients,
6 and there was a significant association between methylation status and mRNA expression level¹³.
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8 NPR1 locus was also found to be differentially methylated in hepatitis C virus-related
9 hepatocellular carcinoma, and its methylation was associated with better prognosis¹⁴. Thus,
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11 NPR1 has influence over blood pressure and other aspects of the cardiovascular system, the
12 behavioral response to itch, the production of pro-inflammatory cytokines in the kidneys, its
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14 ligand ANP has influence over cancer recurrence following surgery in patients with lung cancer,
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16 and methylation of the NPR1 locus can be found in patients with gastric cancer and
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18 hepatocellular carcinoma.

19 We found, by mining multiple independently published microarray datasets, that NPR1
20 was among the genes most differentially expressed in the primary tumors of patients with non-
21 small cell lung cancer; NPR1 was expressed at significantly lower levels in NSCLC tumors
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23 as compared to the lung, and decreased expression of NPR1 was significantly associated with
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25 worse overall survival in NSCLC patients. NPR1 may be of relevance to the biology of tumor
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27 initiation or progression in patients with NSCLC of the adenocarcinoma type, the most common
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type of the leading cause of cancer death in the United States and worldwide.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
40	32625_at	1.95E-16	-15.844297	27.4823646	0.7497 ± 0.0446	NPR1	natriuretic peptide receptor 1

Table 1: NPR1 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 NPR1 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
187	7905606	1.81E-21	-11.860331	38.299373	0.8454 ± 0.0698	NPR1	natriuretic peptide receptor 1

Table 2: NPR1 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 NPR1 expression in NSCLC tumors as compared to the lung, and gene are listed in this chart.

Rank	ID	p-value	t	B	Gene
404	A_23_P147711	4.69E-10	-9.16	13.038685	NPR1

Table 3: NPR1 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.

Figure 1: NPR1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of NPR1 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 NPR1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: NPR1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of NPR1 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 NPR1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: NPR1 expression in NSCLC tumors significantly correlates with overall survival.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=1925$ total patients stratified into two groups, based on low or high expression of NPR1 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 NPR1 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)
52	104

Table 3: NPR1 expression in NSCLC tumors significantly correlates with patient survival.

The median overall survival of $n=1925$ NSCLC patients based on stratification into low or high expression of NPR1 in tumors is listed in this chart.