1 **Tyrosine kinases Tie1 and Tie2 are differentially expressed in non-small cell lung cancer.**

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6 Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States¹.
7 We mined published microarray data^{2,3,4} to identify differentially expressed genes in NSCLC.
8 We found that the genes encoding the tyrosine kinase with immunoglobulin and epidermal
9 growth factor homology domains 1 - Tie1, and its counterpart Tie2 - were both among the genes
10 whose expression was most quantitatively different in tumors from patients with NSCLC as
11 compared to the lung. Tie1 and Tie2 may be important for initiation or progression of non-small
12 cell lung cancer in humans.
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25 Keywords: Tie1, Tie2, NSCLC, non-small cell lung cancer, systems biology of NSCLC, targeted
26 therapeutics in NSCLC.
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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
10 tyrosine kinases Tie1 and Tie2 in adenocarcinoma tumors from patients with NSCLC, suggesting
11 Tie1 and Tie2 may be important for NSCLC tumor initiation or progression.

16 **Methods**

17 We utilized microarray datasets GSE74706², GSE43458³ and GSE33532⁴ and for this
18 differential gene expression analysis of NSCLC tumors in conjunction with GEO2R. GSE74706
19 was generated using Agilent-026652 Whole Human Genome Microarray 4x44K v2 technology;
20 for this analysis, we used $n=18$ control lung tissue and $n=10$ NSCLC tumors, and the analysis
21 was performed using platform GPL13497. GSE43458 was generated using Affymetrix Human
22 Gene 1.0 ST Array technology; for this analysis, we used with $n=30$ control lung tissue and $n=80$
23 NSCLC tumors, and the analysis was performed using platform GPL6244. GSE33532 was
24 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 Tie1 or Tie2 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} to discover in an unbiased fashion and at the transcriptome-level the most striking gene expression features of NSCLC tumors.

Tie1 is differentially expressed in non-small cell lung cancers.

We observed significant differential expression of the tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1) when comparing NSCLC tumors of the adenocarcinoma type to normal lung tissue² (Table 1). When sorting each of the transcripts measured based on significance of difference in expression between NSCLC tumors and the

normal lung, Tie1 ranked 79 out of 34183 total transcripts (Table 1). Differential expression of Tie1 in NSCLC tumors was statistically significant (Table 1; $p=6.18\text{E-}13$).

We queried a second microarray dataset³ to determine if we could validate differential expression of Tie1, and again found significant differential expression of Tie1 when comparing NSCLC tumors of the adenocarcinoma type to normal lung tissue (Table 2). When sorting each of the transcripts measured based on significance of difference in expression between NSCLC tumors and the normal lung, Tie1 ranked 44 out of 33252 total transcripts (Table 2). Differential expression of Tie1 in NSCLC tumors in this second dataset was statistically significant (Table 2; $p=5.69\text{E-}27$).

Tie1 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for Tie1 from NSCLC tumors and from the lung to directly compare Tie1 expression between tumor and control lung tissue and assess for statistical significance. Tie1 was expressed at significantly lower levels in NSCLC tumors as compared to the normal lung in each of the datasets queried (Figure 1 and Figure 2; $p<0.0001$ for each comparison). We calculated a fold change of 0.8458 ± 0.0553 (Table 2) in Tie1 expression when comparing NSCLC tumors to the lung.

Tie1 is one of five receptor tyrosine kinases that display endothelial cell-restricted expression: the vascular endothelial growth factor receptors VEGF-1, VEGF-2, and VEGF-3, as well the counterpart of Tie1, Tie2^{6,7}. Tie1 and Tie2 are both important for embryonic angiogenesis and expressed in vascular endothelial cells during development⁸. Accordingly, we

asked whether Tie2 expression was also perturbed in non-small cell lung cancers.

Tie2 is differentially expressed in non-small cell lung cancers.

We found significant differential expression of Tie2, also known as tunica interna endothelial cell kinase⁶, or Tek, in NSCLC tumors when compared to the lung² (Table 4). When sorting each of the transcripts measured based on significance of difference in expression of Tie2 between NSCLC tumors and the normal lung, Tie2 ranked 61 out of 34183 total transcripts (Table 4). Differential expression of Tie2 in NSCLC tumors was statistically significant (Table 4; $p=3.46E-13$).

Analysis of a second microarray dataset³ again revealed significant differential expression of Tie2 in NSCLC tumors of the adenocarcinoma type (Table 5). When sorting each of the transcripts measured based on significance of difference in expression of Tie2 between NSCLC tumors and the normal lung, Tie2 ranked 31 out of 33252 total transcripts (Table 5). Differential expression of Tie2 in NSCLC tumors was statistically significant (Table 5; $p=1.43E-28$).

We queried a third microarray dataset⁴, again finding significant differential expression of Tie2 in non-small cell lung cancers of the adenocarcinoma type when compared to the normal lung. When sorting each of the transcripts measured based on significance of difference in expression of Tie2 between NSCLC tumors and the normal lung, in this dataset, Tie2 ranked 30 out of 25906 total transcripts (Table 6). Differential expression of Tie2 in NSCLC tumors was statistically significant (Table 6; $p=7.77E-17$).

Tie2 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for Tie2 from NSCLC tumors and from the lung to directly compare Tie2 expression between tumor and control lung tissue and assess for statistical significance. Tie2 was expressed at significantly lower levels in NSCLC tumors as compared to the normal lung in each of the datasets queried (Figure 3, Figure 4 and Figure 5; $p < 0.0001$ for each comparison). We calculated a fold change of 0.7395 ± 0.0904 (Table 4) and 0.6565 ± 0.0645 (Table 5) in Tie2 expression when comparing NSCLC tumors to the lung.

Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed differential expression of the receptor tyrosine kinases Tie1 and Tie2 as among the most significant transcriptional features of NSCLC tumors.

Discussion

Tie2 is an 1122 amino acid receptor tyrosine kinase with an extracellular domain containing fibronectin III repeats and two immunoglobulin-like repeats which are separated by three epidermal growth factor-like repeats. These structural features bear homology to Tie, a human endothelial-specific receptor tyrosine kinase, and thus Tek was named Tie2⁸⁻¹⁰. Studies in transgenic mice bearing null and dominant negative alleles of Tie2 originally indicated that Tie2 played an important role in vasculogenesis, the formation of new blood vessels¹⁰. Homozygous Tie2 embryos at embryonic day 8.5 (E8.5) and E9.0 possess 30% and 75% reductions in endothelial cells, suggesting importance of Tie2 in development of the endothelium¹⁰. Tie2 is

required for interactions between endocardium and myocardium and thus is critical for development of the heart¹⁰. However, later reports directly comparing the role of Tie1 and Tie2 revealed unique requirements for Tie1 and Tie2, indicating that Tie2 is specifically required for angiogenesis, expansion and remodeling of the network of blood vessels that innervate mammalian tissues rather than in endothelial cell development and vasculogenesis⁸. Homozygous Tie2 knockout embryos are dead at E10.5, with defects in formation of the vascular network in the head and the heart⁸. Sprouting and branching appeared to be affected by Tie2 deficiency, with failure to form branching at vessels that serve to conduct myocardial circulation⁸. The ligand for Tie2/Tek is angiopoietin-1 (Ang-1), a secreted protein that can bind Tie2 and induce its phosphorylation on tyrosine residues¹¹. Tie2 is also important for non-embryonic developmental functions such as in maintenance of hematopoietic stem cell quiescence through interactions between Tie2 and Ang-1¹².

Tie2 is implicated in a number of human cancers, in tumors of the breast, the lung, the pancreas and the prostate¹³⁻¹⁷. High expression of Tie2 in “hotspots” could be observed at areas of inflammatory infiltrates at tumor periphery in tumors of patients with invasive breast cancer¹³, and the frequency of Tie2+ blood vessels (CD31+ Tie2+) was significantly increased in breast tumors as compared to normal breast or benign breast tumors¹⁴. Over-expression of Tie2 is significantly correlated with shorter survival in patients with breast cancer¹⁴. Importantly, Tie2 expression was significantly correlated with metastatic risk in breast cancers with lack of spread to the lymph nodes (“node-negative”)¹⁴. Treatment of pancreatic cancer cells with the tyrosine

1 kinase inhibitor Foretinib results in reduction of Tie2 activation, accompanied by increased
2 apoptosis of lymphatic endothelial cells, and Foretinib treatment in Panc-1 pancreatic cancer
3 xenografts resulted in significant decreases in tumor growth and impaired angiogenesis *in vivo*¹⁵.
4 However, conclusions regarding changes in angiogenesis are difficult to draw due to the multi-
5 spectrum nature of kinase inhibitors like Foretinib¹⁵. One study identified a rare population of
6 prostate cancer stem cells that were Tie2⁺; Tie2^{high} cells could be found in prostate cancer cell
7 lines and these cells were capable of metastasizing to the bone¹⁶. Perturbed, higher expression of
8 Ang-1 in the serum and bronchoalveolar lavage fluid (BALF) of patients with NSCLC has been
9 documented¹⁷ but we could not otherwise identify literature describing differential and decreased
10 expression of Tie2 in NSCLC.

16 Tie1 and Tie2 can interact with each other as a heterodimer at the cell surface, and this
17 interaction is inhibitory for Tie2 catalytic activity¹⁸. While Ang-1 can abrogate Tie1/Tie2 complex
18 formation and support Tie2 catalytic function, Ang-2 cannot¹⁸. Not dissimilar to Tie2-null mice,
19 endothelial cell-specific ablation of Tie1 results in rupture of microvasculature and lethality
20 between embryonic days 13.5 and 14.5¹⁹. While Tie1 was important for angiogenesis and for the
21 integrity of embryonic vascular tissues, Tie1 was not required for vasculogenesis¹⁹. A c-fms-
22 Tie1 chimeric protein can be autophosphorylated downstream of Akt and PI 3-kinase, suggesting
23 that Tie1 can functionally transduce a signal²⁰. Ang1 and Ang4 can induce tyrosine
24 phosphorylation of Tie1, but bona fide ligands for Tie1 remained undefined²¹. In 2019,
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investigators reported that the leukocyte cell-derived chemotaxis 2 LECT2 was a ligand for Tie1, directly interacting with Tie1 through the Ig3 domain²². However, LECT2-Tie1 interaction resulted in de-phosphorylation of Tie1 in a dose-dependent manner, while increasing levels of total Tie2 and phosphorylated Tie2²². Moreover, LECT2 stimulation resulted in dissociation of Tie1/Tie2 heterodimers and association of Tie2 homodimers²².

Silencing of Tie1 in human microvascular endothelial cells results in morphological changes suggestive of an endothelial to mesenchymal transition (EndMT); morphological changes were accompanied by down-regulation of vascular endothelial cadherin gene expression, and up-regulation of N-cadherin gene expression²³. In cancer-associated fibroblasts (CAF), expression of S100A4, a mesenchymal marker, was also increased with Tie1 silencing. EndMT phenotype induced by Tie1 silencing was found to be dependent on up-regulation of the zinc finger transcriptional repressor Slug²³. An antibody targeting Tie1 was found to be effective in reducing growth of primary tumors in two pre-clinical mouse models of cancer: of the breast (4T1) and of the lung (Lewis lung carcinoma[LLC]) without resulting in obvious vascular defects in benign tissues. Tie1 blockade using the AB-Tie1-39 antibody also significantly reduced metastasis to lung in the 4T1 model as well as distant metastasis in the LLC model. Thus, Tie1 inhibition appears to be effective in some solid tumor mouse models of cancer in inhibition of primary tumor growth as well as metastasis. It is unclear how the decreased expression of Tie1 we observe here in tumors from patients with NSCLC can be reconciled with tumor and metastasis inhibitory effects of Tie1 in the LLC lung cancer mouse model.

1 We found significant differential expression of the receptor tyrosine kinases Tie1 and
2 Tie2 in primary adenocarcinoma tumors from patients with non-small cell lung cancer, the most
3 commonly diagnosed type of the leading cause of cancer death in the United States. Differential
4 and decreased expression of Tie1 and Tie2 should be validated in separate patient cohorts; Tie1
5 and Tie2 may be of relevance to the biology of NSCLC tumors or as therapeutic targets in
6 NSCLC.
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Rank	ID	p-value	t	B	Gene
79	A_23_P126416	6.18E-13	-1.22E+01	19.5642946	TIE1

Table 1: Tie1 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
44	7900658	5.69E-27	-14.307401	50.794895	0.8458 ± 0.0553	TIE1	tyrosine kinase with immunoglobulin like and EGF like domains 1

Table 2: Tie1 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 Tie1 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
61	A_23_P374695	3.46E-13	-1.25E+01	20.1306889	TEK

Table 3: Tie2 (Tek) 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
31	8154692	1.43E-28	-15.04071	54.420874	0.7395 ± 0.0904	TEK	TEK receptor tyrosine kinase

Table 4: Tie2 (Tek) 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 Tie2 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
30	206702_at	7.77E-17	-16.375983	28.3804586	0.6565 ± 0.0645	TEK	TEK receptor tyrosine kinase

Table 5: Tie2 (Tek) 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 Tie2 expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Figure 1: Tie1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of Tie1 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 Tie1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: Tie1 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of Tie1 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 Tie1 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: Tie2 (Tek) is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of Tie2 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 Tie2 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 4: Tie2 (Tek) is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of Tie2 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 Tie2 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 5: Tie2 (Tek) is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of Tie2 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 Tie2 expression between NSCLC tumors and the lung, a *p*-value, listed above.