The latent transforming growth factor- β binding protein 4, LTBP4, 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 LTBP4 was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. LTBP4 expression was significantly decreased in NSCLC tumors as compared to the lung, and lower expression of LTBP4 in patient tumors was significantly associated with worse overall survival. LTBP4 may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: LTBP4, 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 LTBP4 in
10 adenocarcinoma tumors from patients with NSCLC, suggesting LTBP4 may be important for
11 NSCLC tumor initiation or progression.

12 **Methods**

13 We utilized microarray datasets GSE33532³, GSE43458⁴ and GSE74706⁵ 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 LTBP4 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 tool⁷ for correlation of LTBP4
13 mRNA expression levels with overall survival in non-small cell lung cancer in $n=1925$ 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.

23 LTBP4 is differentially expressed in non-small cell lung cancers.

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26 We found significant differential expression of the gene encoding the latent transforming
27 growth factor- β binding protein 4, LTBP4, in NSCLC tumors when compared to the lung² (Table
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1) When sorting each of the transcripts measured based on significance of difference in expression of LTBP4 between NSCLC tumors and the normal lung, LTBP4 ranked 95 out of 25906 total transcripts (Table 1). Differential expression of LTBP4 in NSCLC tumors was statistically significant (Table 1; $p=-5.41\text{E-}15$).

We queried a second microarray dataset³, to determine if we could validate differential expression of LTBP4 in non-small cell lung cancers. We again found significant differential expression of LTBP4 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 LTBP4 between NSCLC tumors and the normal lung, in this dataset, LTBP4 ranked 185 out of 33252 total transcripts (Table 2). Differential expression of LTBP4 in NSCLC tumors was statistically significant (Table 2; $p=1.6\text{E-}21$).

Analysis of a third microarray dataset⁴ again revealed significant differential expression of LTBP4 in NSCLC tumors of the adenocarcinoma type (Table 3). When sorting each of the transcripts measured based on significance of difference in expression of LTBP4 between NSCLC tumors and the normal lung, LTBP4 ranked 2529 out of 34183 total transcripts (Table 3). Differential expression of LTBP4 in NSCLC tumors was statistically significant (Table 3; $p=1.23\text{E-}05$).

LTBP4 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for LTBP4 from NSCLC tumors and from the lung to directly compare LTBP4 expression between tumor and control lung tissue and assess for

1 statistical significance. LTBP4 was expressed at significantly lower levels in NSCLC tumors as
2 compared to the normal lung in both datasets queried (Figure 1: $p < 0.0001$ and Figure 2:
3 $p < 0.0001$). We calculated a mean fold change of 0.6941 ± 0.0676 (Table 2) in LTBP4 expression
4 when comparing NSCLC tumors to the lung.
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6 LTBP4 expression in NSCLC tumors correlates with overall survival.

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8 We performed Kaplan-Meier survival analysis using LTBP4 mRNA expression in
9 NSCLC tumors coupled with paired overall survival data from each patient, in 1925 NSCLC
10 patients in total, to determine whether LTBP4 tumor expression was correlated with survival
11 outcomes in NSCLC. We found that patients whose tumors expressed lower levels of LTBP4
12 possessed significantly shorter overall survival than patients with high tumor expression of
13 LTBP4 (Figure 3). Median overall survival (OS) of patients in the low expression cohort was
14 56.5 months, while median OS in patients in the high LTBP4 expression cohort was 96 months
15 (Table 4); this difference in median OS based on LTBP4 tumor expression in NSCLC was
16 statistically significant (Figure 3; logrank p -value: 1.6×10^{-10} ; hazard ratio: 0.59 (0.5-0.7)).
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21 Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed
22 differential expression of LTBP4 as among the most significant transcriptional features of
23 NSCLC tumors, and LTBP4 expression was significantly correlated with patient outcomes, as
24 patients with lower tumor expression of LTBP4 possessed significantly worse overall survival.
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Discussion

Transforming growth factor β ligands are generally assembled along with two other molecules: latency-associated peptides (LAPs), as well as latent TGF- β -binding proteins (LTBPs) in structures known as large-latent complexes, from which their release is required for activation; LTBP4 is one of several latent TGF- β -binding proteins. The LTBP4 molecule is expressed most broadly in the heart, uterus, and small intestine, with less prominent expression also observable in the lungs, prostate, testis and colon, and LTBP-4 can be secreted from human lung fibroblasts in culture, and associate with the extracellular matrix⁶. LTBP-4 contains a protease-sensitive hinge region and treatment with either plasmin, chymase, or pancreatic elastase results in liberation of a 220-230 kilodalton fragment⁶. Mice with disruption in the LTBP4 locus, 3C7, develop pulmonary emphysema which worsens with age, as well as cardiomyopathy and colorectal cancer⁷. Elastic fibers in the lungs and colon are fragmented in LTBP4-null mice⁷. Authors concluded that since only the heart, colon, and lungs of LTBP4-deficient mice develop disease, that this suggested tissue-specificity to LTBP4 function⁷. LTBP4-/- fibroblasts produce significantly increased amounts of extracellular matrix, and genes including connective tissue growth factor, plasminogen activator inhibitor-1, and matrix metalloproteinase-2 were all significantly up-regulated⁸. Expression of a dominant negative TGF- β type II receptor lacking the cytoplasmic tail containing the kinase domain that were unable to transduce TGF- β signals did not correct the phenotype of LTBP4-/- fibroblasts, nor did expression of LTBP1, but expression of LTBP4 did, indicating that TGF- β activity was not responsible for this phenotype, but that specific expression of LTBP4 was⁸. BMP-4 signaling

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2 was increased in LTBP1 fibroblasts, BMP-4 could induce expression of matrix proteins, and
3 inhibition of BMP-4 signaling through expression of gremlin partially corrected the matrix
4 phenotype of LTBP4^{-/-} fibroblasts, suggesting BMP-4 was in part responsible for defects
5 observed in LTBP4-deficient fibroblasts⁸. Patients with recessive mutations in LTBP4 display
6 severe respiratory distress, with tracheomalacia and diaphragmatic hernia⁹. All patients with
7 LTBP4 mutations had cutis laxa^{9,10}. In two patients with LTBP4 mutations, candelabra-like
8 fibers from upper dermis were missing. LTBP4 can target microfibrils containing TGF- β and
9 fibrillin-1¹¹, and incorporation of LTBP4 into the extracellular matrix was impaired in two
10 LTBP4 mutant patients⁹. Thus, LTBP4 has essential roles in the structure and function of the
11 extracellular matrix and for fibers in multiple organs.

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16 LTBP4 is important in the lungs. Lungs from LTBP4^{-/-} mice display, among other
17 phenotypes, a large number of defects associated with fiber structure and fibrotic processes,
18 including an absence of an intact elastic fiber network, alveolar septation defects, abnormal
19 extracellular matrix characterized by large bundles of collagen fibers, increased numbers of
20 Sca1^{low} CD49^{high} lung myofibroblasts, reduced angiogenesis and impaired capillarization, and
21 increased ratio of active TGF- β as well as Smad3 phosphorylation¹². TGF- β induction caused by
22 exposure to bleomycin could be attributed to LTBP4, as LTBP4^{-/-} fibroblasts did not up-regulate
23 TGF- β ¹³. Ltbp4 can be expressed as a long or short isoform; mice deficient in the Ltbp4s
24 isoform, Ltbp4s^{-/-}, develop centrilobular emphysema with similarities to late-stage chronic
25 obstructive pulmonary disease (COPD)¹⁴.

1 LTBP4 is implicated in a number of cancers. LTBP4 was suggested to be a metastasis
2 suppressor in hepatocellular carcinoma (HCC); depletion of LTBP4 in HepG2 cells enhanced
3 migration, proliferation and metastasis *in vivo*, and *in vitro* induced gene expression features
4 associated with the epithelial to mesenchymal transition as well as the secretion of TGF- β 1¹⁵.
5 Tumor-secreted TGF- β 1 recruited myeloid-derived suppressor cells, which impaired
6 proliferation of cytotoxic CD8+ T-cells, altogether supported metastasis¹⁵. LTBP4 was expressed
7 at lower levels in HCC tumors, and high expression of LTBP4 was associated with superior
8 survival probability¹⁵. Expression of LTBP4 was decreased in 9 mouse ductal carcinoma *in situ*
9 and in 5 invasive mammary adenocarcinomas¹⁶. Expression of LTBP4 is also decreased in
10 adenocarcinomas and squamous cell carcinomas of the esophagus, and ectopic expression of
11 LTBP4 in OE33 and KYSE180 esophageal cancer cell lines impaired cell migration while not
12 affecting proliferation¹⁷. CpG islands in the promoter of LTBP4L were hypermethylated in the
13 OE33 and KYSE180 esophageal cancer cell lines¹⁷. Contrary to what we found here in multiple
14 datasets, expression of LTBP4 was reported to be up-regulated in lung cancer in one study¹⁸.

20 We found by mining multiple independently published microarray datasets that the latent
21 transforming growth factor beta binding protein 4 was among the genes most differentially
22 expressed in primary tumors from humans with non-small cell lung cancer, with decreased
23 expression of LTBP4 in NSCLC tumors of the adenocarcinoma type. Importantly, we found that
24 LTBP4 expression was significantly associated with overall survival in patients with NSCLC.
25 LTBP4 may be relevant to the biology of non-small cell lung cancer, the leading cause of cancer
26 death in the United States and worldwide.

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Rank	ID	p-value	t	B	FC	Gene	Gene name
95	204442_x_at	5.41E-15	-14.033004	24.2172001	0.7440 ± 0.0429	LTBP4	latent transforming growth factor beta binding protein 4

Table 1: LTBP4 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 LTBP4 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
185	8028872	1.6E-21	-11.883479	38.419829	0.9032 ± 0.0418	LTBP4	latent transforming growth factor beta binding protein 4

Table 2: LTBP4 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 LTBP4 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
2529	A_33_P3221064	1.23E-05	-5.26	2.9581782	LTBP4

Table 3: LTBP4 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.

Figure 1: LTBP4 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of LTBP4 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 LTBP4 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: LTBP4 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of LTBP4 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 LTBP4 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: LTBP4 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 LTBP4 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 LTBP4 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.

Table 4: LTBP4 expression in NSCLC tumors significantly correlates with patient survival.

Low expression cohort (months)	High expression cohort (months)
56.5	96

The median overall survival of $n=1925$ NSCLC patients based on stratification into low or high expression of LTBP4 in tumors is listed in this chart.