FXYD6 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 FXYD6 was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. FXYD6 expression was significantly decreased in NSCLC tumors as compared to the lung, and lower expression of FXYD6 in patient tumors was significantly associated with worse overall survival. FXYD6 may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: FXYD6, 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 FXYD6 in
10 adenocarcinoma tumors from patients with NSCLC, suggesting FXYD6 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 FXYD6 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 FXYD6 mRNA expression levels with overall survival in non-small cell lung cancer in $n=1925$ 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.

FXYD6 is differentially expressed in non-small cell lung cancers.

We found significant differential expression of FXYD6 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 FXYD6 between NSCLC tumors and the normal lung, FXYD6 ranked 79 out of 25906 total transcripts (Table 1). Differential expression of FXYD6 in NSCLC tumors was statistically significant (Table 1; $p=2.76E-15$).

We queried a second microarray dataset³, to determine if we could validate differential expression of FXYD6 in non-small cell lung cancers. We again found significant differential expression of FXYD6 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 FXYD6 between NSCLC tumors and the normal lung, in this dataset, FXYD6 ranked 151 out of 34183 total transcripts (Table 2). Differential expression of FXYD6 in NSCLC tumors was statistically significant (Table 2; $p=6.59E-12$).

Analysis of a third microarray dataset⁴ revealed significant differential expression of an FXYD6-FXYD2 readthrough transcript in NSCLC tumors of the adenocarcinoma type (Table 3). When sorting each of the transcripts measured based on significance of difference in expression of FXYD6 between NSCLC tumors and the normal lung, FXYD6 ranked 322 out of 33252 total transcripts (Table 3). Differential expression of FXYD6 in NSCLC tumors was statistically significant (Table 3; $p=1.72E-18$).

FXYD6 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for FXYD6 from NSCLC tumors and from the lung to directly compare FXYD6 expression between tumor and control lung tissue and assess for statistical significance. FXYD6 was expressed at significantly lower levels in NSCLC tumors as

1 compared to the normal lung in both datasets queried (Figure 1: $p < 0.0001$ and Figure 2:
2 $p < 0.0001$). We calculated a mean fold change of 0.7923 ± 0.0533 (Table 1) in FXYD6
3 expression when comparing NSCLC tumors to the lung.
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6 FXYD6 expression in NSCLC tumors correlates with overall survival.

7 We performed Kaplan-Meier survival analysis using FXYD6 mRNA expression in
8 NSCLC tumors coupled with paired overall survival data from each patient, in 1925 NSCLC
9 patients in total, to determine whether FXYD6 tumor expression was correlated with survival
10 outcomes in NSCLC. We found that patients whose tumors expressed lower expression of
11 FXYD6 possessed significantly shorter overall patient survival than patients with high tumor
12 expression of FXYD6 (Figure 3). Median overall survival (OS) of patients in the low expression
13 cohort was 49.97 months, while median OS in patients in the high FXYD6 expression cohort
14 was 85 months (Table 4); this difference in median OS based on FXYD6 tumor expression in
15 NSCLC was statistically significant (Figure 3; logrank p -value: 1.1×10^{-8} ; hazard ratio: 0.69
16 (0.61-0.79)).
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21 Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed
22 differential expression of FXYD6 as among the most significant transcriptional features of
23 NSCLC tumors, and FXYD6 expression was significantly correlated with patient outcomes, as
24 patients with lower tumor expression of FXYD6 possessed significantly worse overall survival.
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Discussion

The FXYD family is comprised of seven members that share a core FXYD motif, single span transmembrane domains, and type I membrane protein topology with an amino terminal signal sequence^{6,7}. Multiple FXYD proteins including FXYD1 and FXYD7 function in modulating affinity of Na,K-ATPases (NKA) for cations including Na⁺ and K⁺. FXYD6, also known as phosphohippolin, spans 93 amino acids in length, shares 48.1% homology with phospholemman, (FXYD1) and is expressed most broadly in the brain, but with expression also detectable in the lung, testis and colon⁷. FXYD6 is the only FXYD protein expressed in the inner ear⁶. FXYD proteins can function as regulatory subunits of NKA, interacting with $\alpha\beta$ dimers in unique combinations which can reflect tissue-specific expression; these interactions result in altered affinity of NKA for ions being transported⁸. In the hippocampus, cerebellum and SH-SY5Y cells, FXYD6 was found to interact with $\alpha 1$ and $\alpha 3$ NKA subunits⁸. Patch-clamp experiments in oocytes demonstrated that association of FXYD6 with $\alpha 1\beta 1$ or $\alpha 3\beta 1$ subunits decreased NKA current by reducing the number of $\alpha 1\beta 1$ or $\alpha 3\beta 1$ NKA subunits at the plasma membrane as well as increasing the rate at which $\alpha 1\beta 1$ dimers were turned over. In oocytes expressing human $\alpha 1\beta 1$ /NKA, FXYD6 was capable of modulating K⁺ in the context of hyperpolarized membrane potential, while reducing Na⁺ affinity⁹.

FXYD6 has been implicated in human disease. One report described association of single-nucleotide polymorphisms at the FXYD6 locus with risk of development of schizophrenia¹⁰. Expression of FXYD6 is perturbed in some human cancers. In hepatocellular carcinoma (HCC), the expression of FXYD6 is up-regulated in a number of HCC cell lines

1 including HepG2, SNU449, Hep3B and Huh-7 as compared to a control human liver cell line
2 L02¹¹. Depletion of FXYD6 using small interfering RNA (siRNA) in the HepG2 cell line
3 impaired cell migration as well as proliferation *in vitro*, and association of FXYD6 with NKA in
4 HepG2 cells was confirmed¹¹. Interestingly, FXYD6 was able to modulate Src signal
5 transduction in HepG2 cells, with tyrosine phosphorylation at residue 418 decreasing with
6 siRNA depletion of FXYD6¹¹. In an HCC xenograft model, FXYD6 inhibition using antibody
7 blockade with the FD10 antibody was capable of inhibiting tumor growth of HepG2 cells in nude
8 mice as manifested by decreased tumor volume, while mice grafted with SMMC7221 cells stably
9 expressing FXYD6 bore increased tumor weight¹¹. FXYD6 expression is also increased in
10 cholangiocarcinoma as compared to normal bile duct tissue, with higher positive expression of
11 FXYD6 in well- and moderately-differentiated cholangiocarcinoma as compared to poorly
12 differentiated cholangiocarcinoma¹². Thus, while we found decreased expression of FXYD6 in
13 NSCLC, increased expression of FXYD6 has been reported in two human cancers.

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19 We found by mining multiple independently published microarray datasets that FXYD6
20 was among the genes most differentially expressed in primary tumors from humans with non-
21 small cell lung cancer, with decreased expression of FXYD6 in NSCLC tumors of the
22 adenocarcinoma type. Importantly, we found that FXYD6 expression was significantly
23 associated with overall survival in patients with NSCLC. We previously reported that FXYD1
24 was among the genes most differentially expressed in NSCLC¹³. FXYD6 may be relevant to the
25 biology of non-small cell lung cancer, the leading cause of cancer death in the United States and
26 worldwide.
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Rank	ID	p-value	t	B	FC	Gene	Gene name
79	217897_at	2.76E-15	-14.385489	24.8788186	0.7923 ± 0.0533	FXYP6	FXYP domain containing ion transport regulator 6

Table 1: FXYP6 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 FXYP6 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
151	A_23_P150394	6.59E-12	-1.11E+01	17.2465295	FXVD6

Table 2: FXVD6 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 FXVD6 expression in NSCLC tumors as compared to the lung, and gene are listed in this chart.

Rank	ID	p-value	t	B	Gene	Gene name
322	7951977	1.72E-18	-10.566398	31.52881	FXYD6-FXYD2/// FXYD6	FXYD6-FXYD2 readthrough///FXYD domain containing ion transport regulator 6

Table 3: An FXYD6-containing transcript 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: FXYP6 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of FXYP6 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 FXYP6 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: FXYP6 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of FXYP6 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 FXYP6 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: FXYD6 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 FXYD6 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 FXYD6 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)
49.97	85

Table 4: FXYD6 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 FXYD6 in tumors is listed in this chart.