EMP2 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 EMP2⁵ was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. EMP2 expression was significantly decreased in NSCLC tumors as compared to the lung, and lower expression of EMP2 in patient tumors was significantly associated with worse overall survival. EMP2 may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: EMP2, 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} to compare global gene expression profiles of NSCLC tumors to
9 that of the normal lung. We found recurrent and significant differential expression of EMP2 in
10 adenocarcinoma tumors from patients with NSCLC, suggesting EMP2 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 EMP2 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 EMP2
13 mRNA expression levels with overall survival in $n=1144$ 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 EMP2 is differentially expressed in non-small cell lung cancers.

26 We found significant differential expression EMP2 in NSCLC tumors when compared to
27 the lung² (Table 1). When sorting each of the transcripts measured based on significance of
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1 difference in expression of EMP2 between NSCLC tumors and the normal lung, EMP2 ranked
2 66 out of 25906 total transcripts (Table 1). Differential expression of EMP2 in NSCLC tumors
3 was statistically significant (Table 1; $p=1.13\text{E-}15$).

5 We queried a second microarray dataset⁴ to determine if we could validate differential
6 expression of EMP2 in non-small cell lung cancers (Table 2). We again found significant
7 differential expression of EMP2 in NSCLC tumors of the adenocarcinoma type when compared
8 to the normal lung (Table 2). When sorting each of the transcripts measured based on
9 significance of difference in expression of EMP2 between NSCLC tumors and the normal lung,
10 EMP2 ranked 217 out of 33252 total transcripts (Table 2). Differential expression of EMP2 in
11 NSCLC tumors was statistically significant (Table 2; $p=1.08\text{E-}20$).

15 Analysis of a third microarray dataset³ revealed significant differential expression of
16 EMP2 in NSCLC tumors of the adenocarcinoma type. When sorting each of the transcripts
17 measured based on significance of difference in expression of EMP2 between NSCLC tumors
18 and the normal lung, in this dataset, EMP2 ranked 242 out of 34183 total transcripts (Table 3).
19 Differential expression of EMP2 in NSCLC tumors was statistically significant (Table 3;
20 $p=4.18\text{E-}11$).

23 EMP2 is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

25 We obtained exact mRNA levels for EMP2 from NSCLC tumors and from the lung to
26 directly compare EMP2 expression between tumor and control lung tissue and assess for
27 statistical significance. EMP2 was expressed at significantly lower levels in NSCLC tumors as
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1 compared to the normal lung in each dataset queried (Figure 1: $p < 0.0001$, Figure 2: $p < 0.0001$,
2 Figure 3: $p < 0.0001$). We calculated a mean fold change of 0.7627 ± 0.0472 (Table 1) and
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4 0.8673 ± 0.0584 (Table 2) in EMP2 expression when comparing NSCLC tumors to the lung.

6 EMP2 expression in NSCLC tumors correlates with overall survival.

7 We performed Kaplan-Meier survival analysis using EMP2 mRNA expression in NSCLC
8 tumors coupled with paired overall survival data from each patient, in 1144 NSCLC patients in
9 total, to determine whether EMP2 tumor expression was correlated with survival outcomes in
10 NSCLC. We found that patients whose tumors expressed lower levels of EMP2 possessed
11 significantly shorter overall survival than patients with high tumor expression of EMP2 (Figure
12 4). Median overall survival (OS) of patients in the low expression cohort was 57 months, while
13 median OS in patients in the high EMP2 expression cohort was 127 months (Table 4); this
14 difference in median OS based on EMP2 tumor expression in NSCLC was statistically
15 significant (Figure 4; logrank p -value: $1.0e-13$; hazard ratio: 0.42 (0.33 -0.53)).
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20 Thus, blind comparative transcriptome analysis of non-small cell lung cancers using
21 published microarray data revealed differential expression of EMP2 as among the most
22 significant transcriptional features of NSCLC tumors; we found that EMP2 expression was
23 significantly correlated with patient outcomes, as patients with lower tumor expression of EMP2
24 possessed significantly worse overall survival.
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Discussion

EMP2 (epithelial membrane protein-2), a tetraspan protein, was cloned as part of family of membrane glycoproteins containing four transmembrane domains^{5,8}. EMP2 localizes to lipid rafts in association with glycosylphosphatidyl inositol-anchored proteins (GPI-APs); in the presence of 1% Brij-58, EMP2 is found in some but not all membrane fractions overlapping with caveolin-1; despite this overlap on biochemical fractionation, EMP2 does not co-localize with caveolin-1⁸. Ectopic expression of EMP2 results in significant down-regulation of caveolin-1 levels and less significant but 10-fold reduction in caveolin-2 levels⁸. In addition, 3T3 cells expressing EMP2 undergo significantly increased cell death as evidenced by propidium iodide and annexin V staining, and bear significantly increased levels of GPI-AP on the plasma membrane⁸. A separate study also reported EMP2 regulation of caveolin-1 expression, finding caveolin-1 in plasma membrane, nuclear and microsomal fractions in 3T3/V cells; 3T3/EMP2 cells displayed similar fractionation patterns of caveolin-1 but with decreased levels in the microsomal fraction⁹. Decreased expression of caveolin-1 in 3T3/EMP2 cells was independent of proteasomal degradation but attributable to degradation by lysosomal proteases⁹.

In mice, EMP2 expression can be observed at highest levels in the lung, at moderate levels in the eye, heart, thyroid and uterus, and at lower levels in the brain, liver, skeletal muscle, ovary, and submaxillary gland, as well as in peritoneal B1 B-lymphocytes¹⁰. In Balb/c mice injected intraperitoneally with MV cells, a malignant variant of the DAC splenic lymphocyte cell line, retroviral expression of EMP2 resulted in a significant increase in tumor-free animals,

1 indicating EMP2 could suppress tumor formation in one cellular context in mice *in vivo*¹⁰. In
2 low-serum conditions, 4% of 3T3 cells expressing endogenous levels of EMP2 underwent cell
3 death; 55% of cells expressing low levels of EMP2 and 100% of cells expressing high levels of
4 EMP2 underwent cell death that was blocked by caspase inhibitors Z-VAD-FMK and Z-DEVD-
5 FMK¹⁰. Whole-exome sequencing revealed mutations in EMP2 in patients with childhood-
6 onset nephrotic syndrome¹¹. In zebrafish, emp2 is expressed in glomeruli and pronephric renal
7 tubules, and morpholino oligonucleotide depletion of emp2 (in conjunction with p53 depletion to
8 prolong survival) in zebrafish larvae resulted in pericardial effusion¹¹. In the kidneys of adult
9 rats, EMP2 is also expressed in glomeruli, in the podocyte cytoplasm, but not in tubules; EMP2
10 was expressed in endothelial cells in the nucleus¹¹. In podocytes and in human umbilical vein
11 endothelial cells (HUVEC), depletion of EMP2 resulted in increased caveolin-1 protein levels
12 and decreased cell proliferation¹¹.

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17 EMP2 has described functions in the eye. 10% of patients with retinal detachment
18 possess proliferative vitreoretinopathy (PVR), involving changes in cell contraction and
19 associated forces¹². In an in vitro model of PVR utilizing the ARPE-19 retinal pigment epithelial
20 cell line in collagen gel, expression of EMP2 resulted in increased contraction in collagen gel,
21 while depletion of EMP2 resulted in decreased contraction¹². Changes in contraction in collagen
22 gels could not be attributed to changes in expression of integrins, or in migration or invasion;
23 rather, levels of activated but not total focal adhesion kinase (FAK) were significantly increased
24 upon expression of EMP2 in ARPE-19 cells¹². Administration of a FAK/Src inhibitor to
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1 ARPE-19 cells *in vitro* resulted in decreased contraction in a dose-dependent manner in collagen
2 gels, together suggesting that EMP2 functioned through FAK to modulate contractility in retinal
3 pigment epithelial cells¹². Another study of EMP2 function in the eye, investigating pathological
4 corneal neovascularization using an *in vivo* alkali burn model in the mouse cornea, found that
5 administration of an EMP2 antibody resulted in decreased neovascularization with reduced
6 thickness of the corneas, decreased expression of CD34 and CD31, surrogate markers of
7 neovascularization, as well as decreased expression of vascular endothelial growth factor
8 (VEGF)¹³. In human corneal limbal epithelial (HCLE) cells, *in vitro* EMP2 antibody blockade
9 resulted in decreased EMP2 expression 12-24 hours post-administration and decreased VEGF
10 expression 12 hours post-administration, with rebound in both EMP2 and VEGF expression by
11 48 hours¹³. Thus, EMP2 can control expression and localization of caveolin-1, cell proliferation
12 in podocytes and HUVEC, integrity of the glomerular filtration barrier, cell survival through
13 caspase-dependent cell death, contractility in the retinal epithelium and neovascularization in the
14 cornea.

20 EMP2 is implicated in a number of human cancers. In a survey of EMP2 expression in
21 human breast cancers, EMP2 was highest in metastases, significantly lower in invasive ductal
22 carcinoma, even lower in ductal hyperplasia, and lowest in normal breast tissue¹⁴. In breast
23 cancer cell lines, EMP2 expression was detected in triple-negative MDA-MD-468 cells as well
24 as triple-positive ZR-75-1 cells¹⁴. EMP2 antibody administration could induce cell death in
25 MDA-MB-468 and ZR-75-1 cells, and administration of EMP2 antibody *in vivo* in BALB/c mice
26 injected with MDA-MD-468 cells significantly reduced tumor volume but not in mice injected

1 with the Ramos B-lymphoma cell line that does not express EMP2¹³. In the 4T1 lung metastasis
2 mouse model, EMP2 antibody blockade resulted in significant reduction in metastasis burden in
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4 6 out of 8 mice¹⁴. In MDA-MB-231 cells, ectopic expression of EMP2 enhanced cell migration
5 *in vitro* while depletion reduced cell migration¹⁴. Increased cell migration resulting from EMP2
6 expression was concurrent with increased levels of activated FAK and Src¹⁴. In the HEC1A/
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8 EMP2 endometrial cancer xenograft model, administration of EMP2 antibody significantly
9 reduced tumor volume and improved overall survival¹⁵. Depletion of EMP2 in HEC1A/EMP2-
10 grafted BALB/c nude mice decreased vascularization as assessed by CD34+ blood vessel
11 counts¹⁵. In a Boyden chamber assay using HUVEC, EMP2 expression enhanced invasion and
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13 in scratch-wound healing test, EMP2 expression enhanced wound closure as assessed by
14 migration of HUVEC¹⁵. Moreover, capillary tube formation was significantly decreased in
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16 HUVEC grown on low growth factor matrigel when treated with supernatant from HEC1A/
17 EMP2 cells treated with EMP2 antibody in hypoxic but not normoxic conditions¹⁵. EMP2 could
18 support VEGF expression as depletion of EMP2 in HEC1A cells resulted in decreased VEGF
19 levels¹⁴. EMP2 expression was increased in glioblastoma (GBM) cell lines and in a panel of 50
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21 GBM tumors as compared to normal brain tissue, with high expression of EMP2 in GBM tumors
22 significantly correlating with decreased patient survival¹⁶. Similar patterns of EMP2 control of
23 angiogenesis were found in glioblastoma, with EMP2 expression promoting growth of
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25 intracranial U87MG tumors, whereas EMP2 antibody blockade reduced tumor burden and
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27 CD34+ vascularization in athymic nude mice, and EMP2 depletion decreased levels of cell
28 migration, number of vessels formed and VEGF-A expression in T98, U118, and U87MG

glioblastoma cell lines¹⁷. In T98 and U118 but not U87MG cell lines, EMP2 expression supported phosphorylation of STAT3¹⁷. Higher expression of EMP2 was also observed in meningiomas as compared to normal brain tissue and positively correlated with VEGF-A mRNA expression¹⁸. In the A549 non-small cell lung cancer cell line, depletion of EMP2 resulted in increased migration and EMP2 expression could be repressed by sphingosylphosphorylcholine (SPC)¹⁹. SPC induced phosphorylation of keratin 8 (K8) at serine 431 at in A549 cells, an event associated with viscoelasticity of cancer cells²⁰, and depletion of EMP2 in A549 cells resulted in increased levels of K8 S431, both in the presence and absence of SPC²⁰. Modification of K8 phosphorylation involved the protein phosphatase 2A (PP2A); EMP2 expression induced caveolin-1 expression, leading to sequestration of PP2A and its subsequent ubiquitination. Thus, EMP2 expression is described as increased in multiple cancers, its expression supports angiogenesis and cell migration, and its decreased expression or blockade is associated with improved survival in humans (GBM)¹⁶ or in murine cancer models *in vivo*, respectively¹⁵.

We found, by mining multiple independently published microarray datasets, that EMP2 was among the genes most differentially expressed in the primary tumors of patients with non-small cell lung cancer; EMP2 was expressed at significantly lower levels in NSCLC tumors as compared to the lung, and decreased expression of EMP2 was significantly associated with worse overall survival in NSCLC patients. EMP2 may be of relevance to the biology of tumor initiation or progression in patients with NSCLC of the adenocarcinoma type, the most common 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
66	225078_at	1.13E-15	-14.866504	25.7607542	0.7627 ± 0.0472	EMP2	epithelial membrane protein 2

Table 1: EMP2 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 EMP2 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
217	7999387	1.08E-20	-11.522533	36.538153	0.8673 ± 0.0584	EMP2	epithelial membrane protein 2

Table 2: EMP2 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 EMP2 expression in NSCLC tumors as compared to the lung, and gene are listed in this chart.

Rank	ID	p-value	t	B	Gene
242	A_23_P106682	4.18E-11	-1.02E+01	15.4274614	EMP2

Table 3: EMP2 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: EMP2 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of EMP2 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 EMP2 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: EMP2 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of EMP2 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 EMP2 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: EMP2 is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of EMP2 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 EMP2 expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 4: EMP2 expression in NSCLC tumors significantly correlates with overall survival.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=1144$ total patients stratified into two groups, based on low or high expression of EMP2 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 EMP2 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)
57	127

Table 4: EMP2 expression in NSCLC tumors significantly correlates with patient survival.

The median overall survival of $n=1144$ NSCLC patients based on stratification into low or high expression of EMP2 in tumors is listed in this chart.