The endothelial cell adhesion molecule, ESAM, is differentially expressed in non-small cell lung cancer and associates with patient survival.

Shahan Mamoor¹

¹shahanmamoor@gmail.com

East Islip, NY USA

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 the gene encoding the endothelial cell adhesion molecule, ESAM, was among those whose expression was most different in human NSCLC tumors as compared to the lung. ESAM expression levels were significantly decreased in NSCLC tumors as compared to the lung, and lower expression of ESAM in patient tumors was significantly associated with worse overall survival. ESAM may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: ESAM, endothelial cell adhesion molecule, 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 ESAM in
10 adenocarcinoma tumors from patients with NSCLC, suggesting ESAM may be important for
11 NSCLC tumor initiation or progression.

12 **Methods**

13 We utilized microarray datasets, GSE43458², GSE33532³ and GSE74706⁴ for this
14 differential gene expression analysis of NSCLC tumors in conjunction with GEO2R. GSE43458
15 was generated using Affymetrix Human Gene 1.0 ST Array technology; for this analysis, we
16 used with $n=30$ control lung tissue and $n=80$ NSCLC tumors, and the analysis was performed
17 using platform GPL6244. GSE33532 was generated using Affymetrix Human Genome U133
18 Plus 2.0 Array technology; for this analysis, we used $n=20$ control lung tissue and $n=10$ NSCLC
19 tumors, and the analysis was performed using platform GPL570. GSE74706 was generated
20 using Agilent-026652 Whole Human Genome Microarray 4x44K v2 technology; for this

analysis, we used $n=18$ control lung tissue and $n=10$ NSCLC tumors, and the analysis was performed using platform GPL13497. 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 ESAM 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 tool⁶ for correlation of ESAM mRNA expression levels with overall survival in $n=1925$ non-small cell lung cancer 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.

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

We found significant differential expression of the gene encoding the endothelial cell adhesion molecule, ESAM, in NSCLC tumors when compared to the lung² (Table 1). When

1 sorting each of the transcripts measured by microarray based on significance of difference in
2 ESAM expression between NSCLC tumors and the normal lung, in this dataset, ESAM ranked
3 161 out of 25906 total transcripts, equating to 99.4% differential expression (Table 1).
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5 Differential expression of ESAM in NSCLC tumors was statistically significant (Table 1;
6 $p=3.64E-22$).
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8 We queried a second microarray dataset³ to determine if we could validate differential
9 expression of ESAM in non-small cell lung cancers (Table 2). We found significant differential
10 expression of ESAM in NSCLC tumors of the adenocarcinoma type when compared to the
11 normal lung (Table 2). When sorting each of the transcripts measured based on significance of
12 difference in expression of ESAM between NSCLC tumors and the normal lung, ESAM ranked
13 74 of 33252 total transcripts (Table 2), equating to 99.8% differential expression. Differential
14 expression of ESAM in NSCLC tumors was statistically significant (Table 2; $p=2.01E-15$).
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17 Analysis of a third microarray dataset⁴ again revealed significant differential expression
18 of ESAM in NSCLC tumors of the adenocarcinoma type. When sorting each of the transcripts
19 measured based on significance of difference in expression of ESAM between NSCLC tumors
20 and the normal lung, ESAM ranked 1972 out of 34183 total transcripts (Table 3), equating to
21 94.2% differential expression. Differential expression of ESAM in NSCLC tumors was
22 statistically significant (Table 3; $p=2.6E-06$).
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26 ESAM is expressed at significantly lower levels in NSCLC tumors as compared to the lung.
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28 We obtained exact mRNA levels for ESAM from NSCLC tumors and from the lung to

1 directly compare ESAM expression between tumor and control lung tissue and assess for
2 statistical significance. ESAM was expressed at significantly lower levels in NSCLC tumors as
3 compared to the normal lung in each dataset queried, respectively (Figure 1: $p < 0.0001$ and
4 Figure 2: $p < 0.0001$). We calculated a mean fold change of 0.8517 ± 0.0642 and 0.7852 ± 0.0568
5 in ESAM expression when comparing NSCLC tumors to the lung (Table 1 and Table 2,
6 respectively).
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9 ESAM expression in NSCLC tumors correlates with overall survival.

11 We performed Kaplan-Meier survival analysis using ESAM mRNA expression in
12 NSCLC tumors coupled with paired overall survival data from each patient, in 1925 NSCLC
13 patients in total, to determine whether ESAM tumor expression was correlated with survival
14 outcomes in NSCLC. We found that patients whose tumors expressed lower levels of ESAM
15 possessed significantly shorter overall survival than patients with high tumor expression of
16 ESAM (Figure 3). Median overall survival (OS) of patients in the low expression cohort was
17 54.57 months, while median OS in patients in the high ESAM expression cohort was 107 months
18 (Table 4); this difference in median OS based on ESAM tumor expression in NSCLC was
19 statistically significant (Figure 3; logrank p -value: $6.7e-08$, hazard ratio: 0.63 (0.53 - 0.74); false
20 discovery rate=0.01).
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25 Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed
26 differential expression of ESAM as among the most significant transcriptional features of
27 NSCLC tumors, and ESAM expression was significantly correlated with patient outcomes, as
28 patients with lower tumor expression of ESAM possessed significantly worse overall survival.

Discussion

The endothelial cell adhesion molecule, ESAM, was originally described as a membrane of an immunoglobulin adhesion molecule family with selective expression in endothelial cells. This protein contains a transmembrane domain, a signal sequence and V-type and C2-type immunoglobulin domains⁷. ESAM is expressed in at highest levels in vascular endothelial cells but high expression is also observed in the lung and heart, with peak expression observed in the embryonic vasculature⁷. ESAM localizes to cell-cell junctions and in Chinese hamster ovary (CHO) cells, ESAM could function in homophilic interaction-mediated cell-cell adhesions⁸. ESAM is a marker of fetal liver hematopoietic stem cells in the mouse⁸. Highest expression of ESAM was observed in a Rag- c-kit^{hi} Sca1⁺ HSC fraction, and ESAM expression correlated with HSC activity⁸. ESAM has been described as a marker of HSC activation⁹. ESAM expression is induced in LSK cells following 5-FU treatment, and ESAM^{hi} LSK actively divide in response to 5-FU treatment⁹. In methylcellulose cultures, while ESAM^{lo} LSK contain committed progenitors like CFU-GM and BFU-E, ESAM^{hi} LSK contain a relatively high proportion of CFU-mix: primitive multipotent progenitors⁹. ESAM-deficient mice manifest phenotypes related to xenograft vasculature¹⁰. Both the tumor volume and vascular density of B16F10 melanoma and Lewis lung carcinoma cells is markedly reduced in ESAM-deficient mice¹⁰. Endothelial tube formation and migration in response to basic fibroblast growth factor is reduced in ESAM-deficient endothelial cells¹⁰. The membrane-associated guanylate kinase protein, MAGI-1 is an interaction partner of ESAM¹¹. In human umbilical vein endothelial cells, MAGI-1 co-localizes

with ESAM at cell-cell contacts, and in CHO cells, MAGI-1 recruitment to cell-cell contacts required the presence of ESAM, suggesting ESAM is important for recruitment and anchoring of MAGI-1 to endothelial cell tight junctions¹¹. In ESAM-deficient mice, recruitment of neutrophils to chemically inflamed peritoneum is inhibited by 70% at 2 hours post-stimulation¹². The specific step of neutrophil recruitment affected by ESAM deficiency was found to be extravasation and not rolling or adhesion¹². While ESAM is also expressed on platelets, platelet depletion did not affect neutrophil extravasation, suggesting that endothelial cell ESAM was responsible for the neutrophil extravasation phenotype¹². Similar to the effect of ESAM deficiency on neutrophil extravasation, vascular permeability as measured by leak of intravenously injected Evan's blue dye into the peritoneum in response to thioglycollate stimulation was decreased, as was vascular permeability in response to intradermal VEGF₁₆₅ stimulation¹². Depletion of ESAM by small interfering RNA in mouse bEnd.3 endothelioma cells resulted in 77% reduction in Rho activation, without effect on Rac1 or Cdc42, indicating that ESAM could also influence Rho activation¹². Interestingly, a study comparing the relative contributions of the platelet endothelial cell adhesion molecule (PECAM), junctional adhesion molecule (JAM)-A, vascular endothelial cadherin (VE-cadherin) and ESAM to vascular barrier integrity in mice demonstrated that deletion of ESAM increased vascular permeability in the lung but not in the skin, the brain or the heart, while deletion of PECAM and JAM-A did not change vascular permeability in the lung¹³. Moreover, lethality resulting from VE-cadherin deletion only occurred in the presence of ESAM deletion, suggesting VE-cadherin and ESAM

1 could cooperate to maintain vascular barrier integrity - dual ESAM and VE-cadherin deletion
2 resulted in severe blood coagulation in the lung¹³. Thus, ESAM is important for cell-cell
3 contacts in endothelial cells, can influence vascular permeability, neutrophil extravasation,
4 hematopoietic stem cell function, Rho activation, and tumor xenograft vasculature density.
5 ESAM is expressed in the lungs and is important in conjunction with VE-cadherin for vascular
6 barrier integrity in the lungs. We found one report documenting perturbed expression of ESAM
7 in NSCLC, describing ESAM as a differentially expressed gene in NSCLC¹⁴.

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9 We found, by mining multiple independently published microarray datasets, that the
10 endothelial cell adhesion molecule, ESAM, was among the genes most differentially expressed
11 in the primary tumors of patients with non-small cell lung cancer; ESAM was expressed at
12 significantly lower levels in NSCLC tumors as compared to the lung, and decreased expression
13 of ESAM was significantly associated with worse overall survival in NSCLC patients. ESAM
14 may be of relevance to the biology of tumor initiation or progression in patients with NSCLC of
15 the adenocarcinoma type, the most common type of the leading cause of cancer death in the
16 United States and worldwide.
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Rank	ID	p-value	t	B	FC	Gene	Gene name
161	7952436	3.64E-22	-12.165119	39.882776	0.8517 ± 0.0642	ESAM	endothelial cell adhesion molecule

Table 1: ESAM 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 ESAM 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
74	225369_at	2.01E-15	-14.553983	25.190464	0.7852 ± 0.0568	ESAM	endothelial cell adhesion molecule

Table 2: ESAM 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 ESAM 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
1972	A_23_P47410	2.6E-06	-5.82	4.4894225	ESAM

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

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

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

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

Figure 3: ESAM 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 ESAM 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 ESAM 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)
54.57	107

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